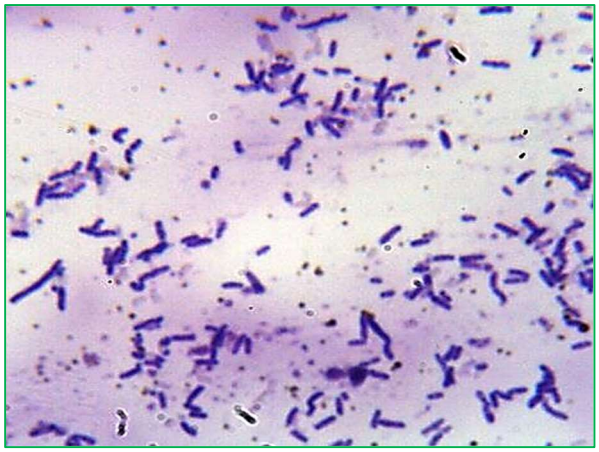
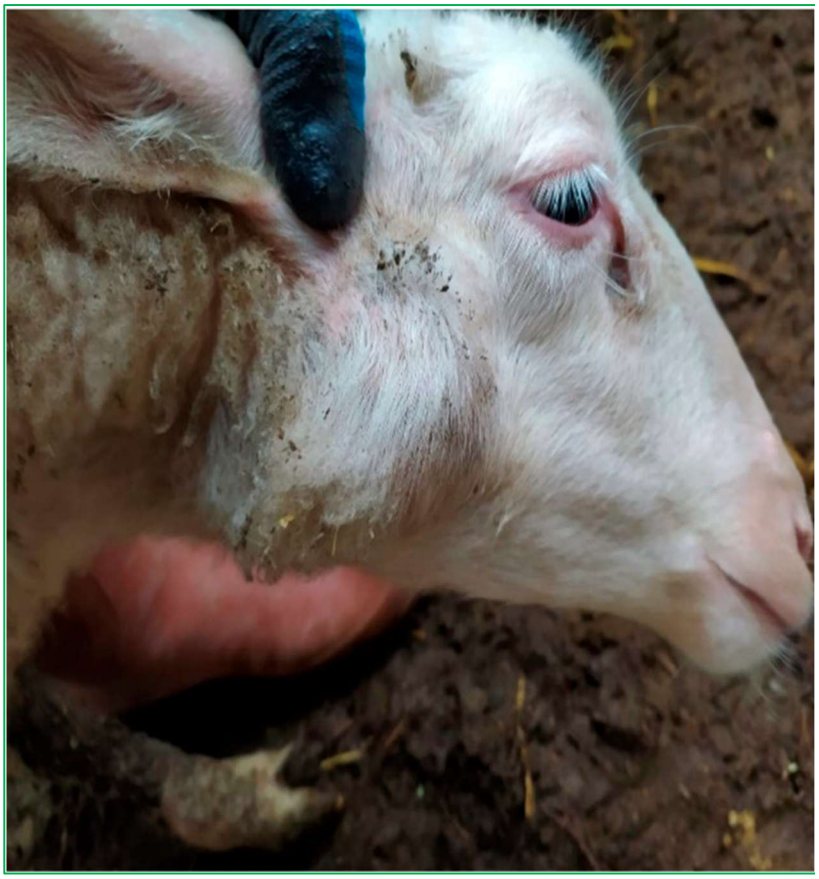
الجمهورية الجزائرية الديمقراطية الشعبية
REPUBLIQUE ALGERIENNE DEMOCRATIQUE ET POPULAIRE
وزارة التعليم العالي و البحث العلمي
MINISTERE DE L'ENSEIGNEMENT SUPERIEUR ET DE LA RECHERCHE SCIENTIFIQUE
جامعة ابن خلدون تيارت
UNIVERSITE IBN KHALDOUN TIARET
معهد علوم البيطرة
INSTITUT DES SCIENCES VETERINAIRES





Pathogenesis and Laboratory Diagnosis of bacterial-Associated Animal Diseases

BY Dr. BOUMEZRAG Assia

2024-2025

TABLE OF CONTENTS

CHAPTER II: GRAM POSITIVE ENDOSPORE-FORMING RODS

CHAPTER III: GRAM POSITIVE NON SPORE-FORMING RODS

LIST OF ABREVIATIONS

API: Analytical Profile Index
BAA: Bile Aesculin Azide Agar
BoNT: Botulinum Neurotoxin
CAMP: Christie–Atkins–Munch-Petersen
CLA: Caseous Lymphadenitis
DNase: Deoxyribonuclease
EF: Extracellular factor
ETA: Exfoliative Toxin A
ETB: Exfoliative Toxin B
Fab: Fragment antigen binding
FAME: Fatty acid–Metabolizing enzyme
Fc: Crystallizable fragment
HCl: Hydrochloric Acid
HE: Haematoxylin and Eosin
Ig : Immunoglobulin
IL: Interleukin
IVS: Institute of Veterinary Science
kDa: kilodalton
MAC: Membrane Attack Complex
MLN: Mesenteric Lympnode
MRP: Muramidase-released protein
MSCRAMM: Microbial Surface Components Recognizing Adhesive Matrix Molecules
MZN: Modified Ziehl-Neelsen
N: Normality
NANAT: Nalidixic Acidnovobiocin- Actidione (cycloheximide)-Potassium Tellurite
SBA: Sheep Blood Agar
SNAP: Synaptosomal-Associated Protein
SNARE: Soluble N-ethylmaleimide-sensitive factor activating protein receptor
SpA : Staphylococcal protein A
TeNT: Tetanus Neurotoxin
TNF: Tumor Necrosis Factor
TNFR: Tumor Necrosis Factor Receptor
TSST: Toxic Shock Syndrome Toxin
VH : Heavy chain Variable domain
vWF: von Willebrand factor

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

LIST OF FIGURES

INTRODUCTION

Several major groups of bacteria are considered to be very important for animal health and welfare. Some of these bacteria are highly pathogenic and are commonly associated with infectious diseases in livestock, pets and wildlife.

The pathogenesis of bacterial infections in animals involves complex host-pathogen interactions, including entry, immune evasion, tissue invasion, and production of virulence factors. Therefore, understanding host-pathogen interactions helps to improve treatment outcomes.

In veterinary bacteriology, it is very important to identify the nature of the bacteria causing infection in animals. Thus, a wide range of laboratory protocols, methods and techniques have been developed to facilitate the identification of the unknown bacteria that are the causative agents of the infection.

Laboratory diagnosis is a multi-step process that includes clinical assessment, specimen collection, microscopy, bacterial culture, biochemical testing, molecular methods and antibiotic susceptibility testing. The combination of these diagnostic tools helps veterinarians accurately identify bacterial pathogens and tailor treatment plans to ensure animal health and prevent further spread of infection.

Specimens for laboratory diagnosis include a wide variety of specimens such as blood, urine, feces, milk, semen, nasal discharge, wound or abscess swabs, aborted fetuses, biopsy specimens and necropsy specimens.

In most laboratory investigations, direct microscopy is the first step to be considered, as the results of Gram staining will help to select further tests to identify the pathogen.

Pure culture techniques are very important and are required to perform various tests. In pure culture, it is possible to obtain a single type of bacterial colony. This facilitates the identification of the unknown microorganism.

The use of biochemical tests in bacterial identification is very helpful and it is an important segment in the diagnostic laboratory. Biochemical tests include carbohydrate, amino acid and lipid metabolism. In addition, these tests depend on the presence or absence of specific bacterial enzymes.

Antimicrobial susceptibility testing is very important to study the development and spread of bacterial resistance and to control the use of antibiotics in veterinary medicine to prevent resistance.

This handout is intended primarily for graduate and undergraduate students in veterinary medicine and for laboratory staff in veterinary bacteriology laboratories. It illustrates the diagnostic aspects of important gram-positive bacteria that cause serious infections in many species (cattle, horses, sheep, goats, dogs, cats, and poultry) with emphasis on appropriate laboratory techniques used for their diagnosis.

GRAM POSITIVE COCCI

I.1. *Staphylococcus* species

I.1.1 General characteristics

Staphylococci are gram-positive cocci (0.5 to 1.5 μm in diameter) that occur singly, in pairs, tetrads, short chains (three or four cells), and in irregular clusters or bunches of grapes. Nearly 50 species and subspecies were known by the early twenty-first century, and new taxa and names continue to be described. They are facultative anaerobes, catalase positive, oxidase negative, non motile and non spore forming **(Quinn et al., 1994; Gyles et al., 2004).**

I.1.2. Classification

Based on the coagulase test, the genus *Staphylococcus* was originally divided into the coagulase-positive species *S. aureus* and the coagulase-negative staphylococci. Coagulase production, or the ability to clot rabbit plasma, remains the most widely used and generally accepted criterion for the identification of pathogenic staphylococci associated with acute infections **(Gyles et al., 2004).**

Three staphylococcal species are of major pathogenic importance in veterinary medicine: *S. aureus*, *S. intermedius* and *S. hyicus*. The first two species are coagulase positive, whereas the coagulase production of *S. hyicus* is variable but mainly negative or weak **(Gyles et al., 2004)**. Table 1 summarizes the animal species in which the various staphylococcal species are found.

Table 1. Staphylococcal species with established pathogenicity in farm and pet animals **(Gyles et al., 2004).**

Animal species	*S. aureus*	*S. intermedius*	*S. hyicus*	Other (in mastitis)
Cattle	x		x	x
Horse	x	x	x	
Pig	x		x	
Sheep	x			x
Goat	x			x
Dog		x		
Rabbit	x			
Mink		x		
Chicken	x		x	
Turkey	x		x	
Pigeon	x	x		

I.1.3. Natural Habitat

Staphylococci are found worldwide in mammals. They colonize the nasal cavity, skin, and mucous membranes and may be transient in the intestinal tract. Staphylococci are closely associated with animals and cannot be considered saprophytes **(Quinn et al., 1994)**.

I.1.4. Bacterial virulence factors

Several potential virulence factors have been described as important in staphylococcal infections. Most of these factors have been studied in Staphylococcus aureus, but some have also been found in Staphylococcus intermedius and other species. The virulence factors can be divided into cell-associated components, exoenzymes and exotoxins **(Fig. 1).**

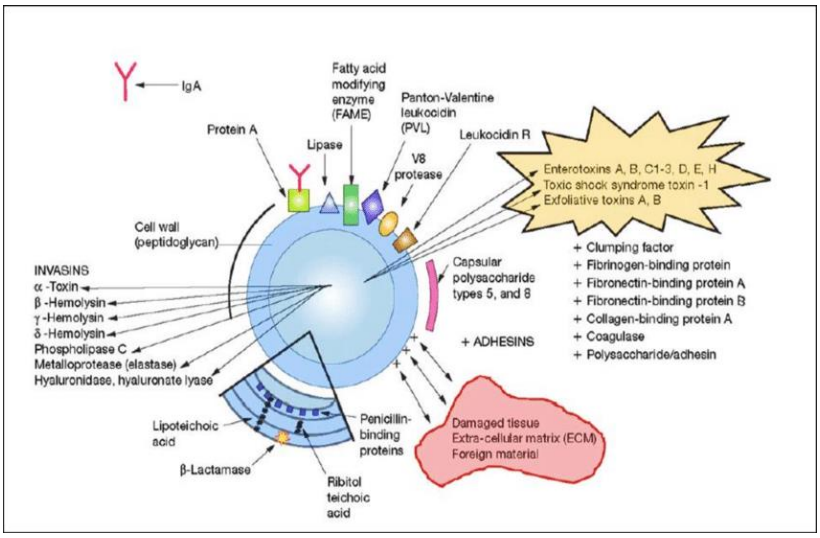


Figure 1. Virulence Factors of *Staphylococcus aureus* **(Daum and Spellberg, 2012).**

I.1.4. 1. Cell-associated components

1. Protein A

Staphylococcal protein A (SpA) is a key virulence factor that enables S. aureus to evade innate and adaptive immune responses. It is a surface protein that binds to the Fcγ portion of human and animal IgG, a defense mechanism that protects S. aureus from opsonization and phagocytic killing. In addition, SpA associates with the Fab portion of VH3-type IgM; cross-linking of B-cell receptors leads to B-cell activation, clonal expansion and subsequent apoptotic collapse, a mechanism that suppresses adaptive immune responses during staphylococcal infection **(Kim et al., 2012)**. Protein A has also been shown to have other properties that modify biological responses. For example, it binds to von Willebrand factor (vWF), an important glycoprotein that mediates platelet adhesion at the site of endothelial damage. In this way, Protein A facilitates *S. aureus* adhesion to vWF-coated surfaces such as endovascular catheters **(Maren von Köckritz-Blickwede, 2009).**

Protein A can also stimulate inflammation in the lung by binding to a receptor for tumor necrosis factor 1 (TNFR-1), which is widely expressed on the airway epithelium. This interaction has been found to play a central role in the pathogenesis of staphylococcal pneumonia **(Fig. 2).**

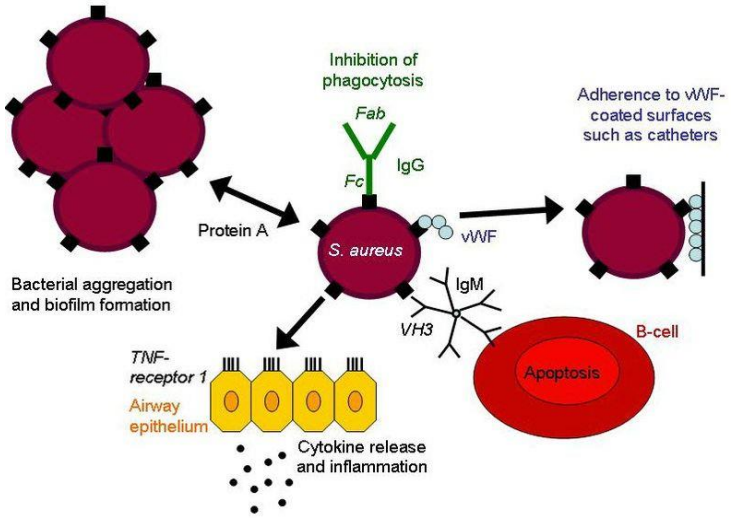


Figure 2. Multifunctional virulence factor Protein A (**By Maren von Köckritz-Blickwede, 2009)**

2. Capsular Polysaccharide

The majority of strains of *S.aureus* are characterized by the presence of capsules, which are defined as slime layer polysaccharides. *Staphylococcus aureus* capsular antigens are surface-associated, limited in antigenic specificity, and highly conserved among clinical isolates. The capsule impedes the process of phagocytosis and enhances the adhesion of the organism to both host cells and prosthetic devices **(O'Riordan and Lee, 2004).**

3. Peptidoglycan and Lipoteichoic Acid

Peptidoglycan, a component of the bacterial cell wall, contributes to the rigidity of the cell wall and activates the complement system. However, staphylococci exhibit a high degree of resistance to complement-mediated lysis because the presence of a thick peptidoglycan layer in their cell wall hinders the insertion of the membrane attack complex (MAC) into the inner membrane **(Pelczar et al., 2007).**

Teichoic acids play a pivotal role in staphylococcal adhesion and colonization, cell division, and biofilm formation. These acids induce septic shock through the same pathways as endotoxin (lipopolysaccharide) in Gram-negative bacteria. Lipoteichoic acids play a role in the induction of septic shock by inducing the production of cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) from macrophages **(Swoboda et al., 2010).**

4. Adhesins

Staphylococcal bacteria may express proteins on their surface that facilitate adhesion to host proteins. These proteins are known as MSCRAMM, and they include fibronectin, laminin, vitronectin, and collagen. These proteins form the extracellular matrix of epithelial and endothelial surfaces. Furthermore, certain strains express a fibrinogen-binding protein (clumping factor), which facilitates adhesion to blood clots and traumatized tissue **(Gyles et al., 2004).**

I.1.4. 2. Exoenzymes

1. Coagulase

Coagulase is an extracellular protein that binds to prothrombin in the host, forming a complex known as staphylothrombin. This complex activates the protease activity, leading to the conversion of fibrinogen to fibrin and bacteria could protect themselves from phagocytic and immune defenses by causing localized clotting **(Projan, 1997).**

2. Staphylokinase (fibrinolysin):

Breaks fibrin clot and allow the spread of infection to contiguous tissues **(Projan, 1997).**

3. Lipase

In response to an infection, the host can produce a variety of fatty acids and other lipid molecules that act as surfactants, thereby disrupting the bacterial membrane, particularly during the formation of an abscess. It has been hypothesised that lipases and an enzyme known as fatty acid–metabolizing enzyme (FAME) produced by *S. aureus* may have a deleterious effect on immune function **(Gyles et al., 2004).**

4. Hyaluronidase

Hyaluronidase and hyaluronate lyase are a family of enzymes that digest hyaluronic acid and are associated with virulence. It has been suggested that depolymerization of hyaluronic acid present in connective tissue contributes to the infectious process by promoting spread through tissue degradation **(Gyles et al., 2004).**

5. Proteases

Proteases have been proposed to block the action of antibodies by cleaving and inactivating them. A second role for proteases may be to protect against antimicrobial peptides such as neutrophil defensins or platelet microbicidal proteins. These proteases may contribute to the destruction of tissue proteins and enhance invasiveness. The V8 protease is responsible for the degradation of fibronectin-binding protein, thereby inducing bacterial spread after the initial adherence step. Another possible role for these proteases is to obtain nutrients from the environment **(Gyles et al., 2004).**

6. DNase

This enzyme degrades DNA and induces tissue injury.

I. 1.4. 3. Exotoxins

1. Enterotoxins and Toxic-shock Syndrome Toxin

Staphylococcus aureus secretes enterotoxins and toxic shock syndrome toxin (TSST-1), two types of superantigenic toxins **(Fig. 3)**.

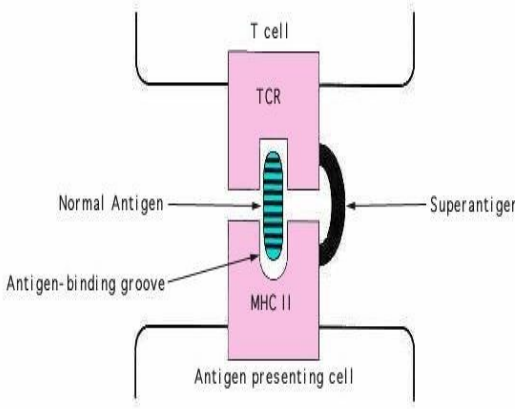


Figure 3. Superantigens and the non-specific stimulation of T cells **(Todar, 2008).**

The role of these toxins has been predominantly described in human medicine, but they have also been observed in cattle, goats, and sheep. Enterotoxins, in particular, have been implicated in the induction of diarrhea and vomiting when ingested. These toxins have been identified as a primary causative agent of staphylococcal food poisoning and have also been associated with the development of TSS when they gain entry into the circulatory system.

TSST-1, a key component of the syndrome, is released into the bloodstream, leading to the onset of toxic shock syndrome (TSS). Superantigens bind directly to class II major histocompatibility complexes (MHC II) of antigen-presenting cells outside of the normal antigen-binding groove. It has been estimated that as many as one in five T cells may be activated, resulting in the release of substantial quantities of cytokines, thereby inducing the manifestations associated with toxic shock syndrome **(Fraser et al., 2000).**

2. Epidermolytic Toxins

The exfoliative toxins ETA and ETB, produced by *S. aureus*, have been demonstrated to induce a range of diseases, from bullous impetigo to scalded skin syndrome in humans, characterized by extensive blistering and epidermal loss.

In contrast, exudative epidermitis in pigs, triggered by virulent *S. hyicus* strains, has been demonstrated to be attributable to toxins that bear a resemblance to *S. aureus* exfoliative toxins in their functional characteristics. However, these toxins are not closely related in terms of immunological responses **(Tanabe et al.,1993).**

3. Haemolysins and Leukocidin

- **Alpha-toxin (α-toxin)**: It is the most thoroughly characterized and potent membrane-damaging toxin of staphylococci. After binding to the toxin, a complex series of secondary reactions occurs, resulting in the release of cytokines that trigger the production of inflammatory mediators **(Gyles et al., 2004).**
- **Beta-toxin (β-toxin):** It is a sphingomyelinase, which damages membranes rich in sphingomyelin. This hemolysin facilitates the recognition of the majority of *S. aureus* strains, though not all, contingent upon the host species with which they are associated. It also permits the identification of virtually all *S. intermedius* strains from dogs on ox or sheep blood agar **(Gyles et al., 2004).**
- **Gamma-toxin (γ-toxin)**, (also referred to as leukotoxin) and leukocidin are proteins that function in tandem to compromise leukocytes and lipid membranes. Few Staphylococcus aureus isolates express leukocidin; however, nearly 90% of the strains from severe dermonecrotic lesions and necrotic pneumonia in humans produce this toxin. This suggests that it is an important factor in necrotizing infections.
- **Delta-toxin (δ-toxin):** It is a very small peptide produced by most strains of *S. aureus*. It has been described to have direct and indirect effects on the activity of neutrophils and monocytes, thereby demonstrating a proinflammatory capacity **(Gyles et al., 2004).**

I.1.5. Pathogenesis

The staphylococci are pyogenic and are associated with abscess formation and suppuration. Pus, a fluid secretion composed of the debris of dead leukocytes, living and dead bacteria, and other components, can be surrounded by intact phagocytic cells, fibrin strands, and eventually surrounded by a fibrous capsule. The pathogenic strains of staphylococci are capable of producing a wide range of toxins and enzymes, which play a pivotal role in the pathogenesis of the bacteria and cause a variety of diseases **(Table 2).**

Table 2. Main diseases caused by pathogenic staphylococci **(Quinn et al., 1994)**

Species	Host(s)	Diseases
Staphylococcus aureus	Many animal species	Abcessess ansd suppurative conditions
	Cattle	Mastitis: subclinical, acute, peracute, chronic or gangrenous Udder impetigo (small pustules often at the base of teats)
	Sheep	Tick pyaemia of lambs (2-5 weeks old) associated with heavy tick infestation (*Ixodes ricinus*) Periorbital eczema (dematitis) Staphylococcal dermatitis: predisposed to by scratches from vegetation such as thistles. Mastitis : acute, peracute or gangrenous. Caseous lymphadenitis
	Goats	Subacute or peracute mastitis Staphylococcal dermatitis
	Pigs	Mastitis: acute, subacute and chronic (botryomycosis). Necrotising stqphylococcal endometritis. Udder impetigo after abrasions from teeth of piglets
	Horses	Acute mastitis Botryomycosis (spermatic cord) after castration
	Dogs, cats	Supputrative conditions similar to those listed for *Staphylococcus intermedius*
	Rabbits	Exsudative dermatitis in neonates Abcessess, conjonctivitis and pyaemic conditions.
	Poultry	Bumble-foot: pyogranulomatous process of subcutaneous tissue of foot that can involve the joints. Staphylococcal arthritis and septicae.
Staphylococcus intermedius	Dogs, cats	Pyoderma Staphylococcal pustular dermatitis under poor hygiene Pyometra External otitis Infections involving respiratory tract, bones, joints, wounds, eyelids and conjunctiva.
Staphylococcus hyicus	Pigs	Exsudative epidemitis (greasy pig disease), usually in pigs under 7 weeks old. There is systemic involvement and the conditions can be fatal. Septic polyarthritis
	Cattle	Rare cases of mastitis

I.1.6. Laboratory Diagnosis

I.1.6.1. Specimens

Potential sample materials may include, but are not limited to, exudates, pus from abscesses, mastitis milk, skin scapings, urine, and affected tissues **(Quinn et al., 2004).**

I.1.6.2. Direct microscopy

Preparation and examination of Gram-stained smears of pus or exudates is often a rewarding process and may yield the presence of Gram-positive cocci of staphylococci in bunches of grape formations **(Quinn et al., 2004).**

I.1.6.3. Isolation

The standard medium for the inoculation of specimens is either nutrient agar or sheep blood agar. Mannitol salt agar and Baird-Parker medium have been demonstrated to be selective for staphylococci. However, the latter is primarily employed in food microbiology. The inoculated plates are then subjected to incubation under aerobic conditions at 37°C for 24 hours **(Quinn et al., 2004).**

Blood agar prepared witheither ovine or bovine erythrocytes is preferable in veterinary diagnostic. Both *S.aureus* and *S.intermedius* are usually hemolytic and often produce both the alpha-lysin and beta-lysin and so exhibit a double zone of hemolysis. The alpha-lysin is responsible of the narrow zone of clear hemolysis immediately around the colony and the beta lysin for the broader outer zone of incomplete partial hemolysis. *S. hyicus* is non-haemolytic.

I.1.6.4. Identification

- **Colonial characteristics**

On nutrient agar, colonies usually appear in 24 hours. They are round, white or slightly yellowish, smooth and glistening but on blood agar, they tend to appear substantial and opaque. On mannitol salt agar, *S. aureus* strains from cattle and other domestic animals usually have a golden-yellow pigments but those from dogs are usually white.

The colonies of *S. intermedius* and *S.hyicus* are also non pigmented. Some of the coagulase negative staphylococci produce pigment especially strains of *S. chromogenes* whose colonies are orange-yellow.

GRAM POSITIVE COCCI

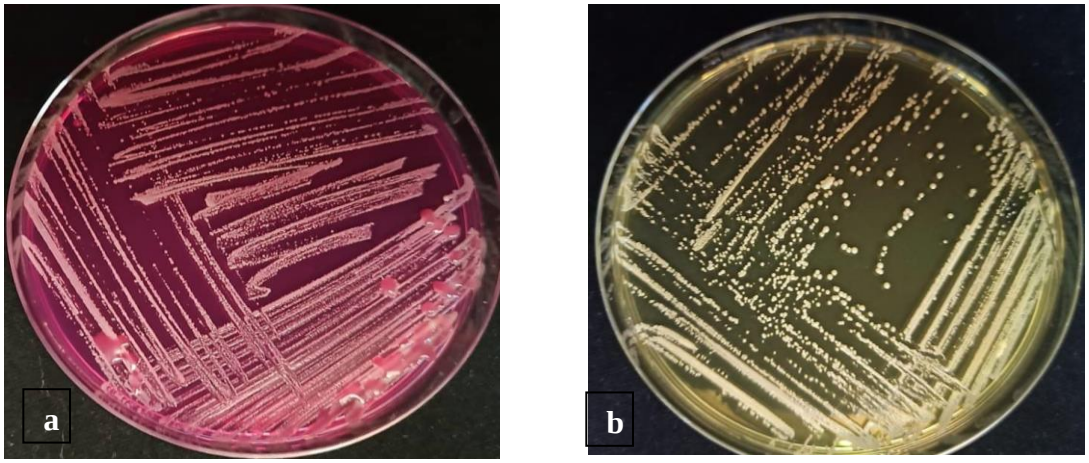


Figure 4. Staphylococci on mannitol salt agar : **a.** non pigmented colonies ; **b.** golden yellowich colonies **(Laboratory of Microbiology, IVS-Tiaret)**

- **Microscopic appearance**

A gram-stained smear from colonies reveals Gram positive cocci randomly distributed over the field and forming irregular grap-like clusters **(Fig.5)**:

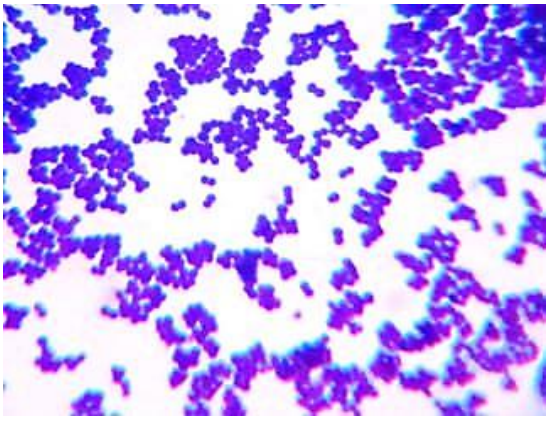


Figure 5. *Staphylococcus aureus* in a Gram-stained smear from a colony (x1000) **(Laboratory of Microbiology, IVS-Tiaret)**

- **Biochemical reactions**

1. **Catalase tests:** the objective of this test is to identify organisms that produce the enzyme catalase. This enzyme plays a pivotal role in the detoxification process by catalyzing the decomposition of hydrogen peroxide into water and oxygen gas. The primary application of the catalase test is the differentiation of staphylococci and streptococci.. The procedure entails the collection of a small quantity of the organism from a well-isolated 18- to 24-hour colony using a sterile inoculating loop, followed by its deposition onto a microscope slide. Then, a dropper or Pasteur pipette is used to place one drop of 3% H_2O_2 onto the organism.

Positive reactions are indicated by the immediate formation of bubbles, as illustrated in Figure 6.

Figure 6. Catalase positive reaction produced by *Staphylococcus* spp**. (Laboratory of Microbiology, IVS-Tiaret)**

2. **Coagulase test:** This test correlates well with pathogenicity. Rabbit plasma contains fibrinogen that is converted to fibrin by the staphylococcal coagulase enzymes. Bound coagulase is detected by the slide test and free coagulase by the tube test.

a. **Slide coagulase test**: A loopful of the staphylococcal culture is emulsified in a drop of water on a microscope slide. Subsequently, a loopful of rabbit plasma is added and thoroughly mixed with the bacterial suspension. The slide is then gently rocked, and a positive reaction is indicated by clumping within one or two minutes **(Fig.7)**

Figure 7. Slide coagulase test: negative staphylococci are present on the left side of the slide, while coagulase-positive staphylococci are present on the right side of the slide **(Katz, 2010).**

b. **Tube coagulase test**: In a small test tube, 0.5 ml of rabbit plasma is placed. Two drops of an overnight broth culture of the staphylococcus or a heavy suspension made from the culture on agar plate in sterile water are then added. The tube is then rotated gently to ensure thorough mixing of the contents, followed by incubation at 37°C, preferably in a water bath. The coagulation of the plasma is indicative of a positive test result, which typically occurs within 2-4 hours. However, it is noteworthy that numerous weak

coagulase-positive strains may require an overnight incubation period to achieve plasma coagulation **(Fig. 8)**

Figure 8. Positive tube coagulase test with clotting of the plasma
(Laboratory of Microbiology, IVS-Tiaret)

3. **DNase test:** The plate should be streaked from the rim to the center in a straight line with a loopful of growth from a blood agar plate. The inoculated plate is then incubated for 18 to 24 hours at 35 to 37°C. Following the incubation period, the plates is flooded with sufficient 1 N hydrochloric acid (HCl). Allow the acid to penetrate the entire medium surface for a duration of five minutes. A clear zone around the colonies demonstrates a positive test after the addition of 1N HCl **(Fig. 9).** A negative test is the absence of a clear zone after the addition of 1N HCl.

Figure 9. *Staphylococcus aureus* from dog otitis showing DNase activity on DNase agar
(Laboratory of Microbiology, IVS-Tiaret)

Table 3 presents a comprehensive summary of the reactions of Staphylococci to coagulase and DNase tests, along with indications of pigment and hemolysin production **(Quinn et al.,1994).**

Table 3. Biochemical reactions and other characteristics of staphylococci isolated from animals

	S.aureus	*S.intermedius*	*S.hyicus*	*S.epidermidis*	*S.saprophyticus*	*S.caprae*	*S.gallinarum*	*S.lentus*	*S.equorum*	*S.simulans*	*S.delphini*	*S.chromogenes*
Coagulase	+	+	d	-	-	-	-	-	-	-	*	-
DNase	+	+	+	d	-	-	-	-	-	-	-	-
Alkaline phosphatase	+	+	+	d	-	(+)	(+)	(±)	(+)	(d)	+	+
Mannitol fermentation	+	(d)	-	-	-	d	-	+	+	+	(+)	d
Maltose fermentation	+	(±)	-	+	-	(d)	+	+	d	(±)	+	d
Pigment production	+	-	-	-	(d)	-	d	d	-	-	-	+
Hemolysis	+	+	-	(d)	-	(d)	(d)	-	(d)	(d)	+	-

+: 90% or more strains positive, ±: 90% or more strains weakly positive, **d:** 11-89% positive **():** delayed reaction, ∗: not known

Commercial systems are also available for the identification of staphylococci such as API Staph **(Fig.10).**

Figure 10. API Staph **(Laboratory of Microbiologiy, IVS- Tiaret)**

I.1.6.5. Antibiotic susceptibility testing

It is imperative that this test be performed on all coagulase-positive isolates and also when a coagulase-negative isolate appears to be significant. The occurrence of resistance to beta-lactam antibiotics is primarily due to a plasmid-encoded penicillinase, also known as a beta-lactamase. In addition, the prevalence of resistance to other antibiotics among staphylococci is a well-documented phenomenon **(Quinn et al., 1994).**

I.2. *Streptococci* and related cocci

I. 2.1. General characteristics

The streptococci and enterococci ae Gram-positive cocci that occur in pairs or chains of varying lengths. They are facultative anaerobes, catalase negative, oxidase negative and non-motile. The streptococci are fastidious and require the addition of blood or serum to the culture media. In contrast, the enterococci are non-fastidious and tolerate the bile salts **(Quinn et al., 1994).**

I. 2.2. Lancefield grouping

The majority of the pathogenic streptococci possess a dominant serologically active carbohydrate that is antigenically distinct from one species or group of species to another. These group-specific cell wall antigens, designated A-H and K-V, can be readily extracted from cell walls by autoclaving, formamide treatment, or enzymic digestion **(Quinn et al., 1994).**

Groups B, C, D, E, G, L, U, and V contain the pyogenic animal streptococci. Notably, certain pathogenic streptococci, such as *S. uberis*, *S. parauberis*, and *S. pneumoniae*, cannot be classified according to the Lancefield scheme. These organisms are identified through various characteristics, including their fermentation behavior, ability to grow at different temperatures, salt tolerance, optochin sensitivity, bile solubility, and 16S rRNA gene sequencing **(Gyles et al., 2004).**

I. 2. 3. Natural habitat

Streptococci are distributed worldwide. The majority of the species of veterinary interest inhabit the mucosa of the upper respiratory and lower urogenital tracts as commensals. These organisms are susceptible to desiccation and have a limited capacity for survival outside of their animal hosts. Enterococci, on the other hand, are classified as opportunistic pathogens and have been identified in the intestinal tracts of numerous animal species **(Quinn et al., 1994).**

I. 2.4. Virulence factors

The virulence of pathogenic streptococci is generally based on surface structures that directly or indirectly block phagocytosis or are involved in adhesion.

The best understood streptococcal virulence factors are the hyaluronic acid capsule and the antiphagocytic M proteins. However, other molecules, including streptolysins, proteases, leukocidal toxins, streptokinase, and possibly surface or secreted plasmin receptors, also contribute to lesion development.

In addition, most pathogenic streptococci have the ability to bind components of the host plasma such as albumin, immunoglobulins, and fibrinogen. Organisms coated with one or more of these components may be able to evade host defenses, either by evading detection or by blocking the deposition of opsonic components of complement **(Gyles et al., 2004).**

GRAM POSITIVE COCCI

Table 4 summarizes the virulence factors of the streptococci most commonly associated with animal disease. With the exception of S. pneumoniae and S. suis, the pathogens listed are often referred to as "pyogenic streptococci" because of their association with pus and purulence.

Table 4. Pathogenic streptococci of animals (**Quinn et al., 1994**; **Gyles et al., 2004).**

Lancefield group	Species	Virulence factor	Natural habitat	Host	Disease
B	*S.agalactiae*	Capsular polysaccharide; C, R and X proteins; CAMP factor; hyaluronidase; lipoteichoic acid; proteases; collagenase; hemolysin; C5a peptidase	Milk ducts	Cattle, sheep and goats	Chronic Mastitis
			Maternal vagina	Dogs	Neonatal septicemia
			Unknown	Cats	Kidney and uterine infection
C	*S.dysgalactiae* subsp. *dysgalactiae*	Hyaluronidase; streptokinase; fibronectin-binding proteins fnb A and B; protein plasminogen receptor; streptodornase; M-like proteins; L2 macroglobulin receptor	Buccal cavity and genitalia	Cattle	Acute mastitis
	S.dysgalactiae subsp. *equisimilis*	As for subsp. *dysgalactiae* but also including streptolysin S and O	Skin and vagina	Horses	Abscesses, endometritis and mastitis
	S.equi	Capsular hyaluronic acid; antiphagocytic SeM; streptolysin S; pyrogenic exotoxins SePE-H and I; peptidoglycan; fibronectin-binding protein; proteases; adhesins; streptokinase; SzPSe protein	Equine tonsils	Horses	Strangles

GRAM POSITIVE COCCI

C	*S.zooepidermicus*	Capsular hyaluronic acid; streptokinase; proteases; streptolysin S; peptidoglycan; SzP protein; fibronectin binding protein; IgG binding protein	vagina skin	Horses	Mastitis Abortion, secondary pneumonia, naval infections
				Cattle	Metritis Mastitis
			Skin and mucous membranes of sows	Pigs	Septicemia and arthritis in 1-3 week-old piglets
			Unknown	Poultry	Septicemia and vegetative endocarditis
			Unknown	Lambs	Pericarditis and pneumonia
	S.equi	Capsular hyaluronic acid; antiphagocytic SeM; streptolysin S; pyrogenic exotoxins SePE-H and I; peptidoglycan; fibronectin-binding protein; proteases; adhesins; streptokinase; SzPSe protein	Equine tonsils	Horses	Strangles
D	*S. suis*	Capsule; MRP and EF proteins; suilysin; Meningo adhesins	Tonsils and nasal cavity	Pigs	Meningitis, arthritis and septicemia
	E. faecalis *E. faecium* *E.durans*	unknown	Intestinal tract	Many species	Septicemia in chickens Bovine mastitis Endocarditis in cattle and lambs and urinary tract infection in dogs
E	*S.pocinus*	M-protein; streptokinase	Mucous membranes	Pigs	Jowl abscesses and lymphadenitis

G	*S.canis*	M protein; streptolysin O	Genital tract and anal mucosa	carnivores	Neonatal septicemia Genital, skin and wound infections
Ungroupable	*S.uberis*	hyaluronic acid capsule that protects against phagocytosis Lactoferrin-binding molecules on the bacterial surface may contribute to attachment to cells	cattle	Skin, vagina and tonsils	Mastitis
	S.pneumoniae	Capsule; neuraminidases; NanA, B; peptidoglycan; teichoic acid; IgA infection protease; adhesins; fibronectin binding proteins	Horses	Upper respiratory tract	Equine respiratory tract

I.2.5. Laboratory diagnosis

I.2.5.1. Specimens

Specimens include exudates, pus, mastitis milk, skin scrapings, cerebrospinal fluid, urine, and affected tissue. Swabs should be submitted in transport media because streptococci are very sensitive to desiccation **(Quinn et al.,1994).**

I.2.5.2. Direct microscopy

Pus, exudate, centrifuged milk or urine smears can be fixed and stained by Gram.

I.2.5.3. Isolation

Routine media include ovine or bovine blood agar. The selective medium is blood agar with 15 milligrams of nalidixic acid and 10 milligrams of colistin sulfate per liter of medium.

Mastitis milk samples may be inoculated on blood agar and Edwards medium. Herd mastitis milk samples may be quarter plated on blood agar containing 0.1 or 0.05 percent aesculin to indicate aesculin hydrolysis**(Quinn et al., 1994).** The selective agar for enterococci is bile aesculin azide agar (BAA). Inoculated plates should be incubated aerobically for 24-48 hours at 37°C.

I.2.5.4. Identification

- **Colonial appearance**

The majority of streptococci manifest as small colonies (approximately 1 mm after 48 hours), while beta hemolytic streptococci produce translucent colonies.

It is noteworthy that colonial variation is a possibility, and mucoid strains of *S. equi* and *S. pneumoniae* are frequently observed **(Fig.11a).** Enterococcus species manifest as small colonies with a brown-black to black halo on BAA medium **(Fig. 11b).**

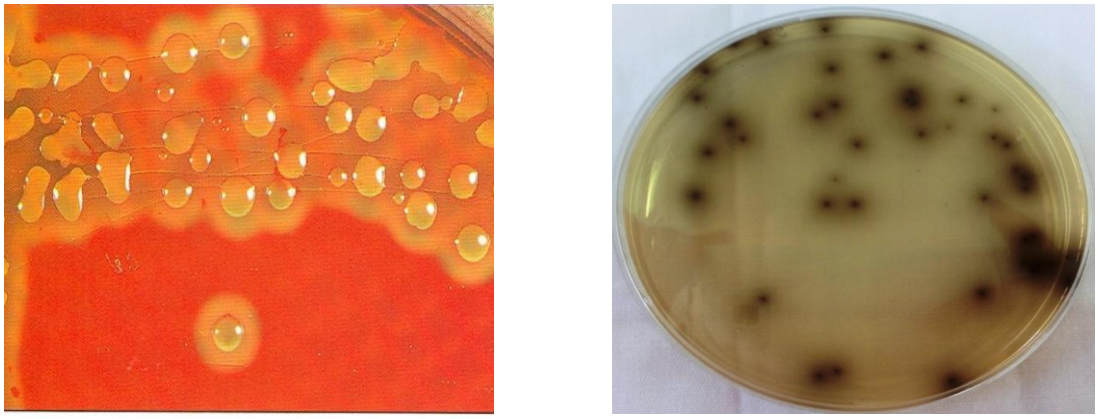


Figure 11. a: Large mucoid colonies and wide zone hemolysis of *S.equi isolated from strangles in a foal* on blood agar **(Quinn et al., 1994); b:** small translucent colonies of *E.faecalis* with black halo on BAA **(Laboratory of microbiology, IVS-Tiaret)**

- **Gram-stained smears**

Gam positive cocci are seen from colonies. The characteristic chains occur only in animal tissues **(Fig.12a)** or in cultures **(Fig.12b).**

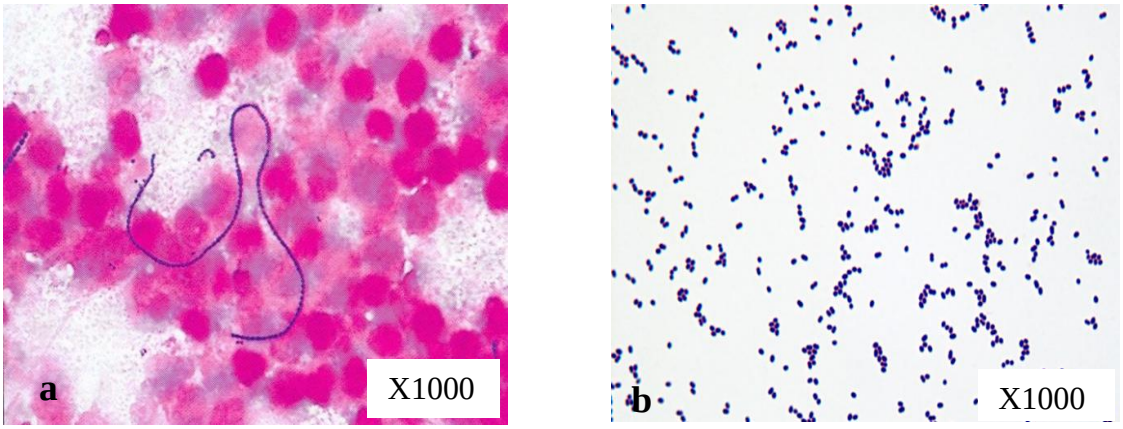


Figure 12. Characteristic chains of Gram-positive cocci, **a:** long chain in a smear of pus from a case of strangles, **b:** short chains from smear of culture **(Quinnet al., 1994)**.

- **Biochemical tests**

1.Catalase test: Streptococci exhibit a negative catalase reaction, a distinguishing characteristic that differentiates them from the catalase-positive staphylococci.

2. CAMP test: A culture of Staphylococcus aureus with a wide zone of partial hemolysis (beta-hemolysin) is streaked across the center of a sheep blood agar plate. Concurrently, a streak of the suspect group B streptococcus is executed at right angles to and taken to within 1 to 1.5 millimeters of the staphylococcal streak. The plate is then subjected to incubation at 37°C for a period of 18 to 24 hours. A positive CAMP test is indicated by an arrowhead of complete hemolysis **(Fig. 13).**

The presence of group B streptococci is indicated by the production of a diffusible metabolite that completes the lysis of partially hemolyzed red blood cells, initiated by the beta-hemolysinof *S. aureus.*

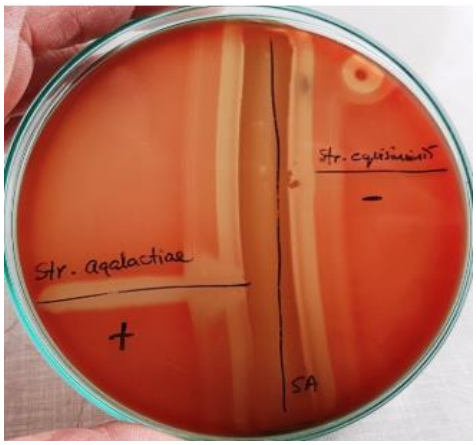


Figure 13. CAMP test for group B streptococci. *S. agalactiae* is causing the characteristic arrow-head clearing of the partial hemolysis (bêta-hemolysis) of *S. aureus* (vertical streak).

3. **Identification using commercial kits such as API strep**:

The API 20 Strep system is a widely used method for identifying streptococcal species. It utilizes a series of 20 biochemical tests to determine the presence or absence of specific enzymatic activities and sugar fermentations, which allows for the identification of most streptococcal and enterococcal species **(Fig.14)**.

Figure 14. API Strep **(Laboratory of Microbiologiy, IVS- Tiaret)**

Table 15 summarises the main biochemical reactions of significant streptococci found in animals:

Table 5. Biochemical reactions of important streptococci from animals **(Quinn and al., 1994)**

	Lancefield group	Acid from: Inulin	lactose	Mannitol	Raffinose	Salicin	Sorbitol	Trehalose	Aesculin	growth in 6.5% NaCl
S .agalctiae	B	-	+	-	-	(+)	-	+	-	-
S. dysgalactiae subsp. *dysgalactiae*	C	-	+	-	-	-	-	+	-	-
S. dysgalactiae subsp. *equisimilis*	C	-	v	-	-	(+)	-	+	-	-
S. equi	C	-	-	-	-	+	-	-	-	-
S. zooepidemicus	C	-	+	-	-	+	+	-	-	-
E. faecalis	D	-	+	+	-	+	+	+	+	+
S. bovis	D	+	+	V	+	+	-	v	+	-
S. suis type 1	D (S)	+	+	-	-	*	-	+	+	-
S. suis type 2	D (R)	(+)	+	-	(+)	+	-	+	+	-
S. porcinus	E	-	(+)	+	-	+	+	+	+	+
S. canis	G	-	(+)	-	-	*	-	(+)	v	-
E. avium	Q	-	+	+	-	+	+	+	+	-
S. uberis	Ung	+	+	+	-	+	+	+	+	(+)
S. pneumoniae	Ung	+	+	-	+	v	-	+	(+)	-

(+): majority of strains positive; **v:** variable reaction; ***:** information not available; **Ung**: ungroupable

- **Serological grouping**

A serological grouping is determined by the C-substance, which is a group-specific cell wall polysaccharide. The following methods are employed to determine the aforementioned grouping **(Quinn et al., 1994):**

1. **Conventional method:** The C substance (antigen) is extracted through either autoclaving or acid extraction (hydrochloric acid). A ring precipitation test is then conducted by layering the extracted antigen over known antisera that can be obtained commercially for each Lancefield group.
2. **Latex agglutination test:** Commercial kits are available for the identification of Lancefield groups A, B, C, D, F and G.
3. **Slide coagglutination Test:** for streptococcal groups A, B, C, F and G.

GRAM POSITIVE ENDOSPORE-FORMING RODS

II.1. *Bacillus* species

II.1.1. General characteristics

Bacillus species are large, gram-positive, spore-forming rods. They are aerobic or facultatively anaerobic, catalase positive and motile with the exception of *Bacillus anthracis* **(Quinn et al., 1994).**

II.1.2. Natural Habitat

Most *Bacillus* species are saprophytes, widely distributed in air, water and soil **(Quinn et al., 1994).**

II.1.3. Virulence factors

The majority of Bacillus species are not considered to be pathogens. However, *Bacillus anthracis,* the causative agent of anthrax, is the most significant animal pathogen in the genus, responsible for the disease in both humans and animals.

The major virulence factors of *B. anthracis*, capsule and toxins, are encoded on two large virulence plasmids, pXO1 and pXO2, both of which are required for full virulence **(Quinn et al., 1994).**

The polypeptide capsule is antiphagocytic, and the tripartite toxin is leukocidal, increasing vascular permeability and causing capillary thrombosis, which can lead to shock.

The toxin gene complex comprises the protective antigen (PA), the lethal factor (LF), and the edema factor (EF) **(Leppla, 1991).** The three exotoxin components combine to form two binary toxins that act as potent enzymes in the cytoplasm of target host cells **(Jones et al., 2019). (Fig.15)**.

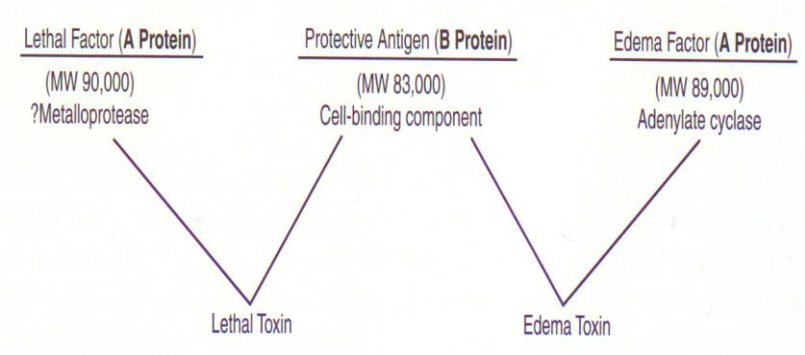


Figure 15. Binary toxins of *Bacillus anthracis* **(Leppla, 1991).**

II.1.4. Pathogenesis

Following internalization within a host, endospores are rapidly phagocytosed by regional macrophages, which subsequently migrate to regional lymph nodes. It has been established that endospores possess the capacity to germinate within the macrophage, subsequently developing

into vegetative bacteria. These vegetative bacteria are released from the infected macrophages and proliferate rapidly in the blood, reaching titers of up to 107–108 per milliliter, resulting in severe bacteremia **(Dixon et al., 2000).**

Bacillus anthracis expresses virulence factors, including toxins, that escape of bacilli from phagosomes and their subsequent release from the infected macrophages **(Dixon et al., 2000).** The resulting toxemia is closely associated with the systemic symptoms of anthrax **(Fig.16)**:

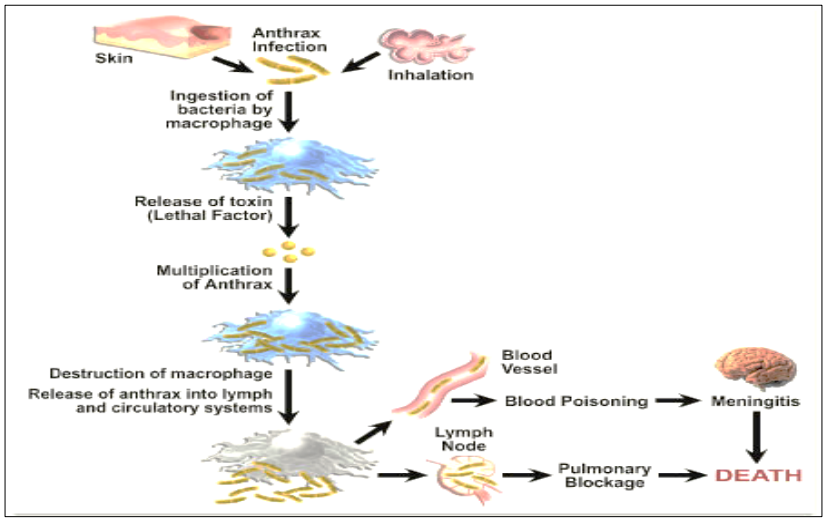


Figure 16. Pathogenesis of anthrax **(Dixon et al., 2000)**

Bacillus anthracis is the causative agent of anthrax, a zoonotic disease of livestock. The primary routes of entry include the ingestion of spores from soil during grazing or the consumption of contaminated foodstuffs, as well as infection through wounds. Inhalation of spores occurs in humans but to a lesser extent in animals. Ruminants are most susceptible to infection, while horses and humans are intermediate, and carnivores and pigs are resistant but may succumb at high infectious doses **(Quinn et al., 1994).**

Table 6. Main diseases and hosts of *Bacillus anthracis* **(Quinn et al., 1994).**

Host	Disease
Cattle and sheep	Septicemic form of anthrax, often accompanied by sudden death
Pigs	Subacute anthrax, marked by the presence of edema in the pharyngeal tissues and frequently accompanied by lymphadenitis adenitis or intestinal form associated with high mortality.
Horses	Oral route: Septicemia with colic and enteritis Wound infections: Localized edema and lymphadenitis
Carnivores	Comparatively resistant. The disease pattern in this species is analogous to that observed in pigs. A substantial dosage resulting from the ingestion of anthrax-infected carcasses can lead to septicemia.

Anthrax is a peracute disease of cattle and sheep that is characterized by septicemia and sudden death. Post-mortem findings include exudation of tarry blood from body orifices, failure of blood to coagulate **(Fig. 17)**, incomplete rigor mortis, and splenomegaly in cattle. In less susceptible species, inflammatory edema of the face, throat, and neck is a common finding, and colic may occur in horses and gastroenteritis in carnivores **(Quinn et al., 1994).**

Figure 17. Tarry and uncloted blood from mouth and nose of cattle died from anthrax Photo provided by Professor Corrie Brown, Lifestock Interntaional (2021)

II.1.5. Laboratory Diagnosis

It is imperative to exercise the utmost caution when handling samples from individuals suspected of being infected with anthrax. All procedures must be carried out in a biohazard safety cabinet, and all infectious and contaminated materials must be autoclaved. It is imperative to note that heat-fixed stained swabs have the potential to pose a significant hazard, as they may contain viable spores **(Quinn et al., 1994).**

II.1.5.1. Specimens

In instances where anthrax is suspected in cattle or sheep, thin blood smears should be prepared from blood drawn from the ear or tail veins. In the case of horses and pigs, collection of edema fluid from localized sites is recommended. Notably, peritoneal fluid is frequently more diagnostically useful than blood smears in pigs. Additionally, the utilization of blood or homogenized spleen samples can be beneficial for culturing purposes, further facilitating the diagnostic process **(Quinn et al., 1994).**

II.1.5.2. Direct microscopy

Bacillus anthracis produces a capsule in vivo and either Giemsa or polychrome methylene blue staining is used to demonstrate the capsule, which is of diagnostic importance. Capsular material is more abundant if the blood smear has been taken from a recently dead animal.

A polychromatic methylene blue-stained smear (M'Fadyean stain) shows blue rods with square ends in chains surrounded by pink capsular material characteristic of *B. anthracis* **(Fig.18).**

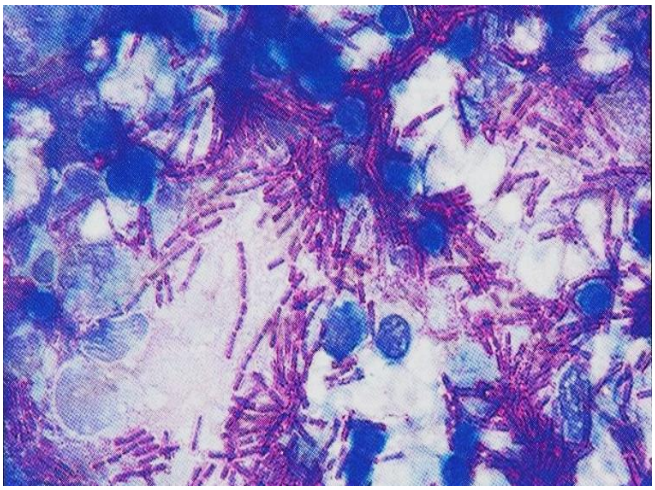


Figure 18. *B. anthracis* in a bovine blood smear collected from a peripheral blood vessel showing square-ended blue rods in short chains surrounded by a pink capsule (M'Fadyean stain, x1000)

II.1.5.3. Isolation

Bacillus anthracis can be isolated on sheep blood agar (SBA), aerobically at 37°C for 24-48 hours.

II.1.5.4. Identification

1. Colonial characteristics

B. anthracis is virtually non-haemolytic. After 48 hours of incubation, flat and dry colonies about 5 mm in diameter are formed. They are greyish to white with a granular, ground-glass appearance. Under low magnification, curved and curled peripheral projections at the edge of the colonies give the appearance of a medusa head **(Fig. 19).**

B. anthracis can produce a capsule when grown on nutrient agar containing 0.7% sodium bicarbonate under 10% CO_2. The colonies are quite mucoid **(Quinn et al.,1994).**

Figure 19. *B. anthracis* on SBA showing non hemolytic, flat and dry ground-glass colonies with irregular edges (Medusa head colonies)

2. Microscopic appearance

A Gram-stained smear of colonies shows Gram-positive rods in long chains (boxcars). Endospores may be produced in older cultures and appear as oval, unstained areas within the mother cell **(Fig.20)**:

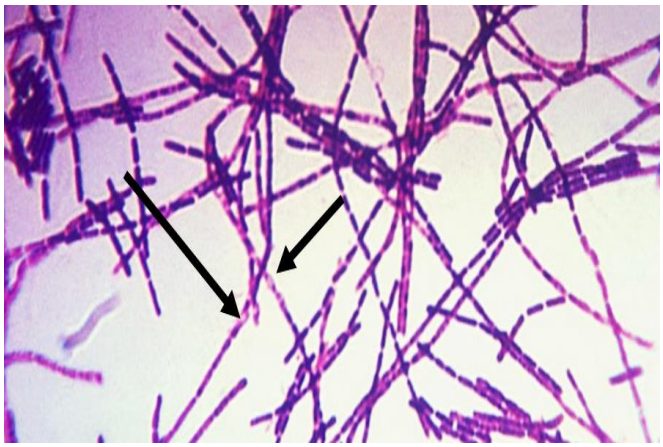


Figure 20. *B. anthracis* from a culture showing gram positive rods in long chains with incipient sporulation (Gram stain, x1000).

3. Biochemical tests

Bacillus anthracis slowly produces an inverted-fi-tree type of gelatin liquefaction with side shoots radiating from the line of the stab **(Fig.21).**

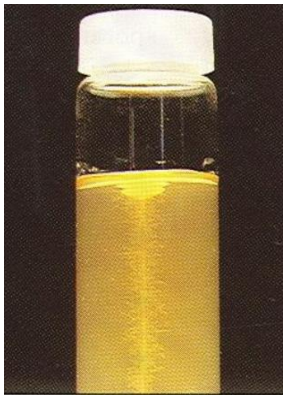


Figure 21. *B. anthracis* stab inoculated into nutrient gelatin giving the characteristic inverted fir-tree type growth after 8 days at 25°C.

II.1.5.5. Antibiotic susceptibility testing.

Bacillus anthracis is very susceptible to penicillin, as well as many other antimicrobials, but usually disease is discovered too late to treat **(Quinn et al., 1994).**

II.2. *Clostridium* species

Clostridium species are large, gram-positive, anaerobic, endospore-forming rods, and the spores usually bulge the mother cell. They are fermentative, oxidase and catalase negative **(Quinn et al., 1994).**

II.2.1. Neurotoxigenic Clostridia

II. 2.1.1. *Clostridium tetani*

Clostridium tetani is an anaerobic, gram-positive, slender, motile bacillus. When it sporulates, the terminal spore bulges the cell giving the organism a characteristic "drumstick" or "tennis racket" shape **(Gerding and Johnson, 2012).**

II.2.1.1.1. Natural habitat

Soil, especially that contaminated by animal faeces, is the natural habitat of *C. tetani*. The spores of this bacterium are ubiquitous in the environment, although their frequent occurrence in horse manure seems to be a myth that has been perpetuated for decades. Spores are concentrated in some geographical areas and are present at much lower levels in other regions **(Quinn et al., 1994).**

II.2.1.1.2. Virulence factors

Clostidium tetani produces two exotoxins:

1. **Tetanolysin**, an oxygen-labile haemolysin that inhibits the chemotaxis of phagocytes and may also increase local tissue damage and promote bacterial proliferation.

2. **Tetanospasmin (TeNT)**, a neurotoxin that is plasmid encoded and acts presynaptically on motor neurons, blocking synaptic inhibition and causing spastic paralysis and the characteristic tetanic spasms **(Fig.22):**

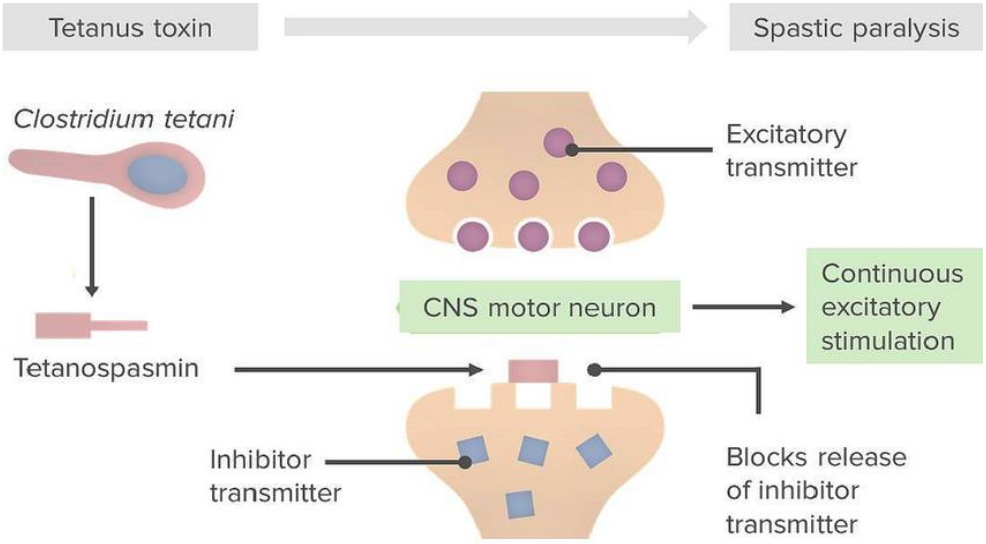


Figure 22. Mechanism of action of tetanus toxin **(Boer et al., 2024)**

II.2.1.1.3. Pathogenesis

Clostridium tetani is a non-invasive bacterium. Therefore, spores or bacteria must enter the animal through a breach in the body's natural barrier. Once inside the tissue, the spores germinate and the bacterium produces the neurotoxin (TeNT) under anaerobic conditions. Portals of entry for *C. tetani* are contaminated traumatic wounds in horses, castration band-induced lesions in sheep, goats and cattle, and tail-docking wounds and dehorning sites in adult cattle **(MacInnes et al., 2022)**.

Anaerobic conditions develop in deep wounds, tissue necrosis and after co-infection with other bacteria. The toxin binds to specific receptors on the motor neuron at the neuromuscular junction. Tetanus toxin reaches the central nervous system after retrograde transport through local peripheral motor nerve terminals **(Deprez et al., 2006).**

Tetanospasmin binds specifically to gangliosides in nerve tissue and, once bound, cannot be neutralized by antitoxin. If the toxin travels up a regional motor nerve in a limb, tetanus develops first in the muscles of that limb, then spreads to the opposite limb and moves upward. This is known as ascending tetanus and is usually seen only in less susceptible animals such as dogs and cats. Descending tetanus is the most common form in susceptible species such as humans and horses. In this form, the toxin circulating in the blood affects the susceptible motor nerve centers

serving the head and neck first, and later the limbs. Once established, the signs of tetanus are similar in all species **(Quinn et al., 1994).**

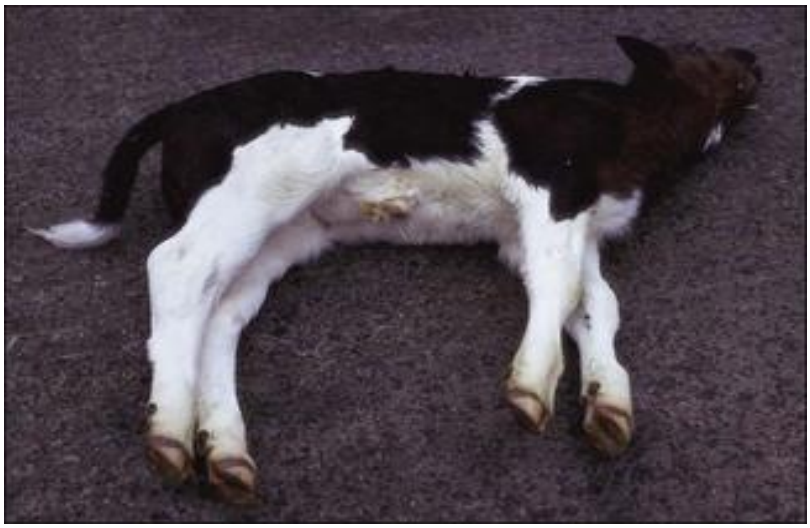


Figure 23. *Clostridium tetani*: advanced tetanus in a young calf showing rigidity of limbs, opisthotonos and raised tail-head. Note pyogenic infection of umbilicus, the probable portal of entry of *C. tetani* in this case **(Quinn et al., 1994).**

II.2.1.1.4. Laboratory Diagnosis

Tetanus is often diagnosed on the basis of history and characteristic clinical signs without reference to laboratory tests **(Quinn et al., 1994):**

1. **Direct microscopy**

Gram stained smears of material from a wound may show the characteristic drumstick spore forms of *C. tetani* **(Fig. 24).** This is not fully diagnostic as other clostridia such as *C.tetanoides* and *C. tetanomorphum* have a similar morphology.

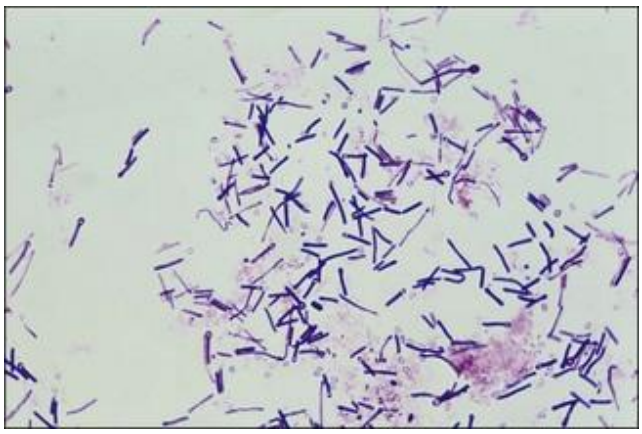


Figure 24. Sporing rods of *C.tetani* in gram-stained smear of necrotic material from a penetrating wound. The spores are spherical, terminal and bulge the mother cell giving the typical drum-stick appearance (Gram stain, x1000); **Quinn et al. (1994).**

2. Isolation

Necrotic tissue from a wound or wound exudate may be heated to 80 °C for 20 minutes and used to inoculate a blood agar plate and another blood agar plate containing 3 % agar (stiff agar). A tube of thioglycollate medium or cooked meat broth may also be inoculated and subcultured on blood agar after two to three days of incubation. Blood agar plates are incubated at 37 °C for three to four days in an atmosphere of H_2 and CO_2 **(Quinn et al., 1994).**

3. Identification

- **Colonial morphology**

Clostridium tetani is hemolytic and on normal blood agar has a tendency to grow in a spreading, swarming pattern **(Fig. 25),** while on 'stiff agar' (3%) it forms single rhizoid colonies **(Fig. 26).**

Figure 25. *Clostridium tetani* colonies on sheep blood agar (1,5% agar) showing swarming growth and a narrow zone of beta-hemolysis **(Ganesh et al., 2016)**

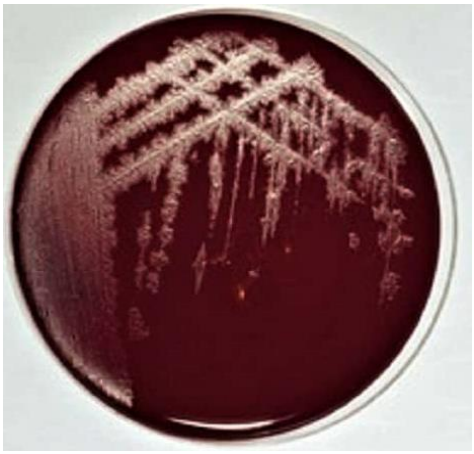


Figure 26. *Clostridium tetani* on stiff sheep blood agar (3% agar) which prevents spreading and gives individual rhizoid colonies **(Ganesh et al., 2016)**.

- **Biochemical reactions**

Clostridium tetani liquefies gelatin; however, it does not ferment the typical range of carbohydrates, including glucose, lactose, sucrose, and maltose. Consequently, the primary focus

of research in this field is on the detection and identification of the toxin rather than on the isolation and identification of the bacterium itself **(Quinn et al., 1994).**

- **Toxin identification**

The presence of toxins in the serum of animals or in filtrates from cooked meat broths or thioglycollate media can be detected in laboratory animals and identified by neutralisation or protection tests using a specific antitoxin. In the protection test, animals are protected with antitoxin at least two hours before inoculation with the toxin-containing material. Typical signs of tetanic spasms are exhibited in the region of inoculation by control mice **(Fig. 27).**

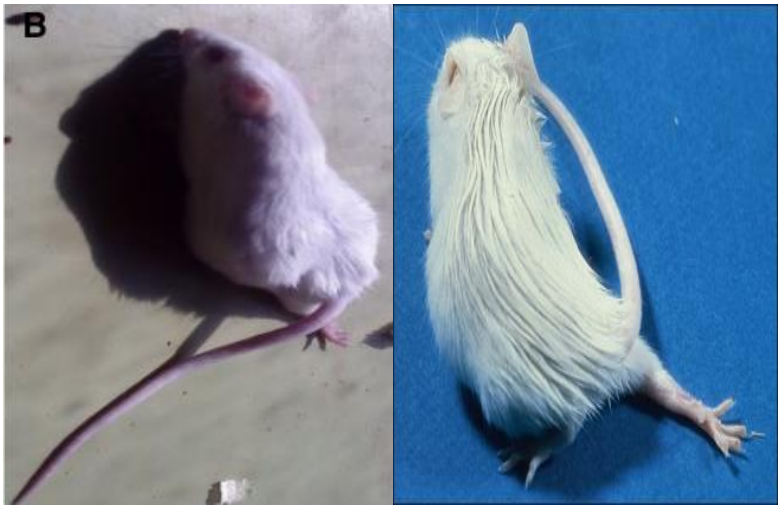


Figure 27. Mouse showing the effects of injected cultured toxin after 24 hours, with stiffness in tails and limbs **(Hanif et al., 2015).**

II.2.1.2. *Clostridium botulinum*

Clostridium botulinum is a straight rod (0.9-1.2 × 4-6 µm) and produces oval, subterminal spores at a pH near or above neutral. The spores are characterised by their exceptional resistance; however, they are susceptible to destruction at temperatures of 121°C for a duration of 15 minutes, while the toxins are destroyed at 100°C for 20 minutes **(Quinn et al., 1994).**

II.2.1.2.1. Classification

Eight distinct neurotoxins are synthesised by *C. botulinum* types A-G (with *C. botulinum* type G now being classified as *C. argentinense*). These toxins are identical in their mechanism of action, yet they vary in terms of their potency, distribution, and antigenicity. Notably, types C and D are recognised as being bacteriophage encoded, underscoring the complexity of toxin synthesis. The optimum pH for C. botulinum is neutral to slightly alkaline (pH 7.0-7.6), and the optimum temperature is between 30°C and 37°C **(Quinn et al., 1994).**

II.2.1.2.2. Natural habitat

The endospores are widely but inconsistently distributed in soils and aquatic environments throughout the world. The germination of the endospores, accompanied by the growth of vegetative cells and the production of toxins, occurs in anaerobic environments, including contaminated tins of meat, fish or vegetables; carcasses of invertebrate and vertebrate animals; rotting vegetation; and baled silage **(Quinn et al., 1994).**

II.2.1.2.3. Virulence factors

Botulinum neurotoxin *(BoNT)* is the primary virulence factor and the causative agent of botulism (**Rawson et al., 2023).** As illustrated in Table 7, the production of toxins by *C. botulinum* types A-E is associated with particular sources and animal species.

Table 7. *Clostridium botulinum* Toxins **(Quinn et al., 1994).**

C. botulinum **Type**	**Toxin produced**	**Source of toxin**	**Susceptible animals**
A	**A**	Fish, meat, fruits and vegetables	Chickens and mink
B	**B**	Vegetables, Fish, meat and meat products often from pigs	Chicken, horse and cattle
C	**C1**	decaying vegetation, invertebrate carcasses	Waterfowls
	C2	baled silage, chicken manure as feed supplement, carcasses and spoiled feeds	Cattle, horses, dogs and mink
D	**D**	Contaminated bones and carcasses of small mammals	Cattle, sheep, horses
E	**E**	Sludge in earth bottomed ponds Fish, fish-products	Fish, birds

II.2.1.2.4. Pathogenesis

Botulism intoxication may occur by ingestion of preformed botulinum neurotoxin (BoNT) or may be toxi-coinfectious (dissemination of toxin from an infected wound or from a focus of *C. botulinum* multiplication in the gastrointestinal tract). Horses are much more susceptible than cattle to BoNT, perhaps due to degradation of preformed toxin by rumen microbes in cattle.

Horses absorb more toxin before it reaches the colon, where microbial degradation occurs in equids **(Mainil, 2006).**

Wound botulism occurs when spores germinate in a wound and produce toxin. In horses, this occurs most often at castration sites, umbilical hernia repairs**.** Wound botulism also occurs in calves castrated by banding **(Mainil, 2006).**

The toxin is absorbed from the intestinal tract and is transported via the bloodstream to peripheral nerve cells where it acts at the neuromuscular junctions of cholinergic nerves and also at peripheral autonomic synapses **(Quinn et al., 1994).**

BoNTs are proteins of approximately 150 kDa, composed of two subunits (light chain and heavy chain) linked by a disulfide.

The heavy chain is involved in binding to the receptor on the neuronal membrane susceptible and the translocation of the light chain into nerve cells **(Uzal et al., 2016).** The light chain is a zinc metalloprotease that acts on synaptobrevin and other SNARE proteins such as syntaxin and Snap 25, interfering with the exocytotic cell mechanism and preventing release of acetylcholine in the synaptic cleft **(Fig.28):**

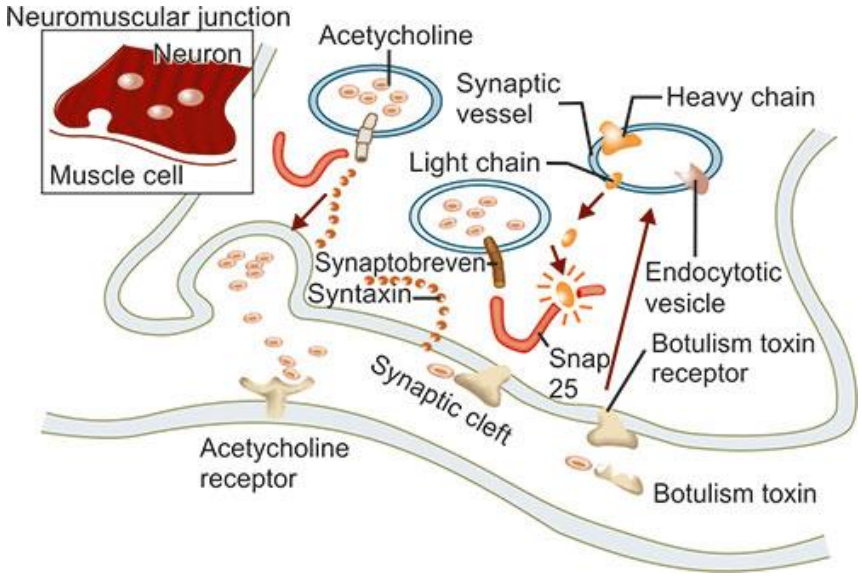


Figure 28. Mechanism of action of botulism toxin **(Trasi Srilata, 2006).**

Botulism is most common in water birds , ruminants, horses, mink and poultry. Carnivores are relatively resistant to all types and pigs are susceptible to the toxin of type A but resistant to those of B, C and D. The oral toxicity of type D toxin is high for cattle and type C toxins are more readily absorbed through the intestinal wall of chickens and pheasants.

Figure 29. Turkey with typical signs of botulism: paralysis of legs, wings, and neck **(Le Maréchal et al., 2016).**

Figure 30. Botulism in cattle; on the left: cow showing clinical signs of sternal recumbency and hind limb weakness, on the right: Tongue paralysis
(Dlabola et al., 2015; Le Maréchal et al.,2016)

II.2.1.2.5. Laboratory diagnosis

The diagnosis of botulism is based on history, clinical signs and demonstration and identification of toxin in serum of moribund or recently dead animals as well as the detection of toxin and/or *C. botulinum* in the suspect foodstuff. Demonstration of toxin in animals that have been dead for some time may not be significant.

C. botulinum spores can be transient in the intestines of normal animals and the death of the animal creates an anaerobic environment suitable for the germination of the spores and toxin production **(Quinn et al., 1994).**

- **Toxin demonstration**

Serum or centrifuged serous exudates from animals can be directly inoculated intraperitoneally into mice. If toxin is present the characteristic 'wasp waist' appearance **(Fig. 31)** in the mice will be seen in a few hours or up to five days.

The appearance is due to abdominal breathing because of paralysis of respiratory muscles. Cattle are extremely susceptible to botulism and detection of toxin in serum is difficult. Thus detection of toxin in gastrointestinal contents, which have been frozen immediately after collection to prevent postmortem multiplication of *C. botulinum* organisms, may be a more rewarding approach (https://veteriankey.com/clostridium-species)

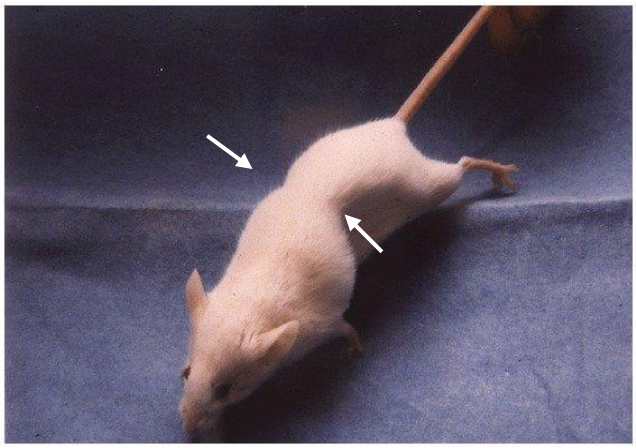


Figure 31. Mouse with classical "wasp waist" sign (diaphagmatic paralysis) after intoxication with botulinum toxin (**Vossen et al., 2012)**.

Extraction of toxin in foodstuffs is accomplished by macerating the product in saline overnight. The suspension is centrifuged and the supernatant filtered through a 0.45 µm bacteriological filter. As the toxin can be in a protoxin form, nine parts of filtrate are treated with one part of 1% trypsin solution and incubated at 37°C for 45 minutes. Mice or guinea pigs can be inoculated intraperitoneally **(Cai and Singh, 2007).**

- **Toxin identification**

Mouse (or guinea pig) neutralization tests using a polyvalent antitoxin initially, followed by monovalent antitoxins, if they are available, are used to identify the toxin and the type of *C. botulinum* involved.

A comparaison of the toxins of the two neurotoxigenic Clostridia is given in the following table:

Table 8 . Comparaison of the toxins of *C. tetani* and *C. botulinum* **(Quinn et al., 1994).**

	Clostridium tetani	***Clostridium botulinum***
Site of toxin production	Wounds	Carcasses, decaying vegetation and occasionally wounds and intestine
Mode of action	The toxin exerts its effect at the central level, cleaving proteins that serve to mediate the fusion of neurotransmitter vesicles with the presynaptic membrane of inhibitory interneurons.	The toxin exerts its effect peripherally. It cleaves proteins that mediate the fusion of neurotransmitter vesicles with the presynaptic membrane of cholinergic nerves.
Type of paralysis	Spastic	Flacid
Antigenic types of toxin	Tetanospasmin (one antigenic type)	Eight different toxins produced by types A to G

II.2.2. Histotoxic Clostridia

II.2.2.1. *Clostridium perfringens*

Clostridium perfringens is common in the environment and in the intestinal tracts of humans, domestic and wild animals. Infecting organisms may be of exogenous origin, but are often endogenous. It is perhaps the most commonly isolated pathogenic bacterium and is undoubtedly the most important cause of clostridial disease in domestic animals **(Mainil, 2006).**

II.2.2.1.1. Classification

Toxin production patterns vary considerably among different strains, providing the basis for a recently revised classification scheme (Table 9) that assigns *C. perfringens* isolates to one of the seven toxin types (A-G):

Table 9. Main toxins produced by *C. perfringens* (**Fu et al., 2022)**.

Toxinotype	**Toxin produced**					
	Alpha (CPA)	**Beta (CPB)**	**Epsilon (ETX)**	**Iota (ITX)**	**CPE**	**NetB**
A	++	-	-	-	-	-
B	+	++	+	-	-	-
C	+	++	-	-	±	-
D	+	-	++	-	±	-
E	+	-	-	++	±	-
F	+	-	-	-	±	-
G	+	-	-	-	-	+

II.2.2.1.2. Natural habitat

Clostridium perfringens is commonly found in decaying vegetation, soil, feces, marine sediment and in the normal gastrointestinal (GI) tract microbiota of humans and other animals (**Mehdizadeh Gohari et al., 2021**, **Fu et al., 2022).**

II.2.2.1.3. Pathogenesis

Clostridium perfringens is a major pathogen of humans and livestock. The virulence of this bacterium can be ascribed, in large part, to its armory of ~20 potent toxins **(Fig. 32, Table 9).**

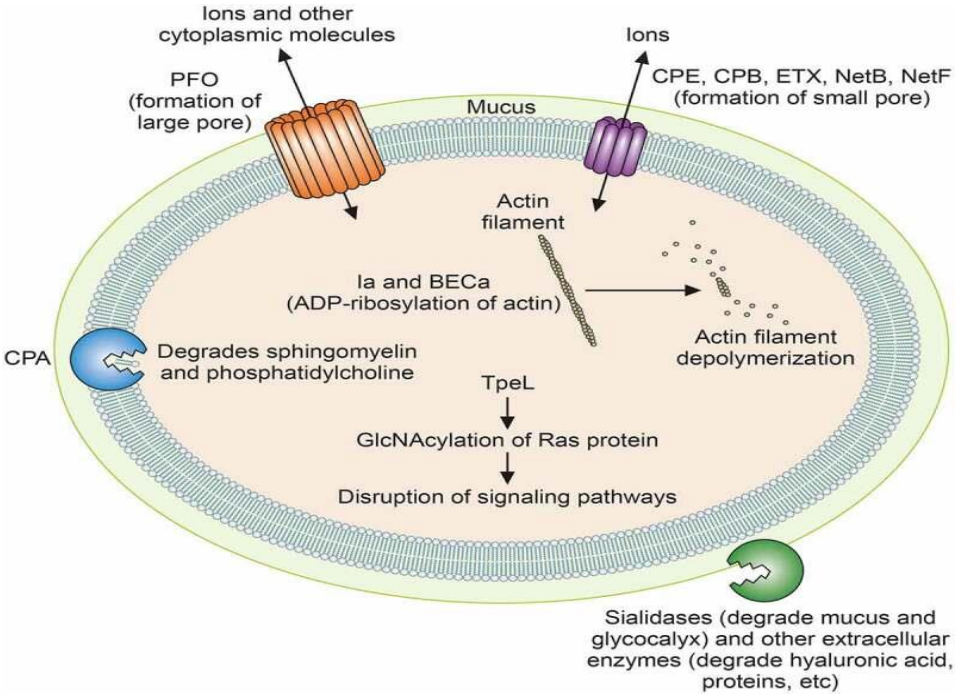


Figure 32. Actions of *C. perfringens* toxins and degradative enzymes **(Mehdizadeh Gohari et al., 2021)**

1. Major Toxins Used for *Clostridium perfringens* Isolate Typing

a. Alpha toxin (CPA):

Alpha toxin is produced by all types and is a multifunctional zinc metalloenzyme consisting of phospholipase C and sphingomyelinase **(Oda et al., 2015).** CPA toxin is encoded by the plc (or cpa) gene located on the chromosome and is the major virulence factor for gas gangrene in humans and clostridial myonecrosis in animals. As gas gangrene progresses, CPA perforates the cell membrane and activates the arachidonic acid cascade and protein kinase C, leading to cellular dysfunction. Meanwhile, CPA suppresses the immune response by preventing leukocytes from entering the infected tissue and reduces the blood supply to infected sites by inducing vasoconstriction, thrombosis and platelet aggregation **(Titball et al., 1999; Fu et al., 2022)**.

b. Beta toxin (CPB):

It has both lethal and necrotizing activities. It is sensitive to trypsin, which explains the predilection of types B and C for neonates, as colostrum has anti-trypsin activity. The beta toxin is the most important factor in the enterotoxaemia caused by type B. At the cellular level, CPB causes necrosis and hemorrhage in the small and large intestine**,** resulting in numerous human and animal diseases **(Uzal et al., 2010)**.

For example, *C. perfringens* type B causes fatal hemorrhagic dysentery in sheep, while type C causes human enteritis necroticans (pigbel), necrotising enteritis and enterotoxaemia in several livestock species **(Navarro et al., 2018).**

c. Epsilon toxin (ETX):

It is released as a weakly active prototoxin and is activated by several intestinal proteases, such as alpha-chymotrypsin, trypsin and carboxypeptidases, which remove N- and C-terminal residues from this protoxin, resulting in a mature, active protein that is 1000 times more toxic than the prototoxin. ETX is thought to increase intestinal permeability, thereby facilitating its own absorption (his effect is not fully understood, but involves the opening of tight junctions in the mucosa and degenerative changes in the junctions and degenerative changes in the intestinal lamina propria **(Goldstein et al., 2009).** Microscopically, lesions in the tend to be multifocal and characterised by perivascular and intramural vascular oedema, and in the brain by hemorrhage and, in more chronic cases, white matter necrosis and swelling of astrocytes and axons **(Uzal et al., 2010).**

The latter is usually symmetrical and bilateral. The origin of these lesions is thought to be the result of initial binding of the toxin to endothelial cells of the blood-brain barrier (BBB). After ETX binding, endothelial cells become swollen, vacuolated and necrotic **(Uzal, 2004).**

Epsilon toxin can be considered an enterotoxin and a neurotoxin. It is mainly produced by *C. perfringens* types B and D **(Navarro et al., 2010).**, which are responsible for enterotoxaemia in goats and sheep, and less commonly in cattle **(Li et al., 2011).**

This toxin also affects organs other than the intestine, such as the heart, central nervous system and lungs , resulting in severe brain lesions, haemorrhages, white matter necrosis and vascular oedema **(Uzal et al., 2010).**

d. Iota toxin (ITX):

is a binary toxin produced only by *C. perfringens* type E strains, is comprised of an enzyme component (Ia) and a binding component (Ib). ITX is produced as protoxin which is then cleaved by by trypsin or chymotrypsin producing an active form.

The lipolysis-stimulated lipoprotein (LSR, also known as angulin-1) is a cellular receptor for Ib **(Hunter et al., 1993).** Once bound to its receptor, the Ib binding component oligomerizes into heptamers that insert into the plasma membrane of target cells to form functional channels, facilitating the movement of ions and the translocation and endocytosis of the Ia enzymatic component **(Garcia et al., 2013).** After endocytosis, Ia translocates from late endosomes into the cytoplasm where it exerts ADP-ribosylating activity involving the covalent attachment of ADP-ribose onto an Arg at residue 177 of actin **(Navarro et al., 2010).**

This effect induces depolymerization of actin filaments, which increases the presence of G-actin monomers **(Navarro et al., 2010).**

By ADP-ribosylating actin, iota toxin changes cell morphology and disorganizes intercellular tight and basolateral junctions, producing an increased paracellular permeability in cultured intestinal cells *in vitro* **(Navarro et al., 2010).** Iota toxin causes cells necrosis by inducing cytotoxic activity, which affects mitochondrial function, ATP reduction, increased inorganic phosphate (IP), and cells swelling, and the toxin is responsible for animal diseases such as hemorrhagic enteritis **(Theoret et al., 2018).**

e. *C. perfringens enterotoxin* (CPE):

CPE is a35 kDa single polypeptide and detected in type C, D, E, and F strains. The CPE protein consists of a C-terminal receptor-binding domain and an N-terminal cytotoxicity domain that mediates oligomerization and membrane insertion during pore formation **(Navarro et al., 2010).** Moreover, this toxin is only expressed during *C. perfringens* sporulation. It is accumulated in the beginning of the sporulation and excreted out when cells lysate at the end of sporulation (**Monturiol-Gross et al., 2014).**

f. Necrotic enteritis B-like toxin (NetB):

This toxin causes cell lysis by osmotic pressure and cell necrosis, inducing avian necrotic enteritis under certain predisposing factors **(Navarro et al., 2010).**

g. Perfringolysin O (PFO):

PFO is produced by most *C. perfringens* strains, with the exception of type F strains. Its main activity is related to alteration of the membrane integrity **(Navarro et al., 2010).**

h. TpeL toxin:

Toxin perfringens large (TpeL), a ~ 205 kDa protein, belongs to the clostridial glucosylating toxin (**Garcia et al., 2013**). TpeL causes an N-acetylglucosaminylation of Ras proteins at threonine 35, thereby inactivating these small GTPases and inducing myriad signaling effects that lead to inflammation and cell death (**Garcia et al., 2013).**

2. Degradative Enzymes

C. perfringens also produces other toxins and degradative enzymes to degenerate tissues, including Theta (θ) toxin, kappa (κ) toxin, proteases, hemolysins, μ toxin, ν toxin and neuraminidase. Theta toxin weakens the host inflammatory response to the infection by destroying blood vessels (hemolysis), breaking down leukocytes, and leading to cardiotoxicity **(Navarro et al., 2010).**

Kappa toxin (collagenase) helps the bacterium's rapid spread from connective tissue plains into deeper muscle tissues by breaking down connective tissue **(Navarro et al., 2010).**

Although mu toxin (hyaluronidase) is a non-lethal toxin, it has the ability to facilitate the spread of α toxin and degrades mucins and connective tissues **(Navarro et al., 2010).** nu toxin (DNase) damages the nuclei of polymorphonuclear leukocytes and muscle cells in gas gangrene **(Navarro et al., 2010).** These toxins and proteins work together to cause necrosis of muscle, subcutaneous tissue, and add in the production of gas with hydrogen.

II.2.2.1.4. *Clostridium perfringens*-Induced Diseases

Unlike other anaerobic bacteria infecting limited animal hosts and their tissues, *C. perfringens* enjoys a successful living spectrum from muscles to the gut of different animal **(Fig.33)**

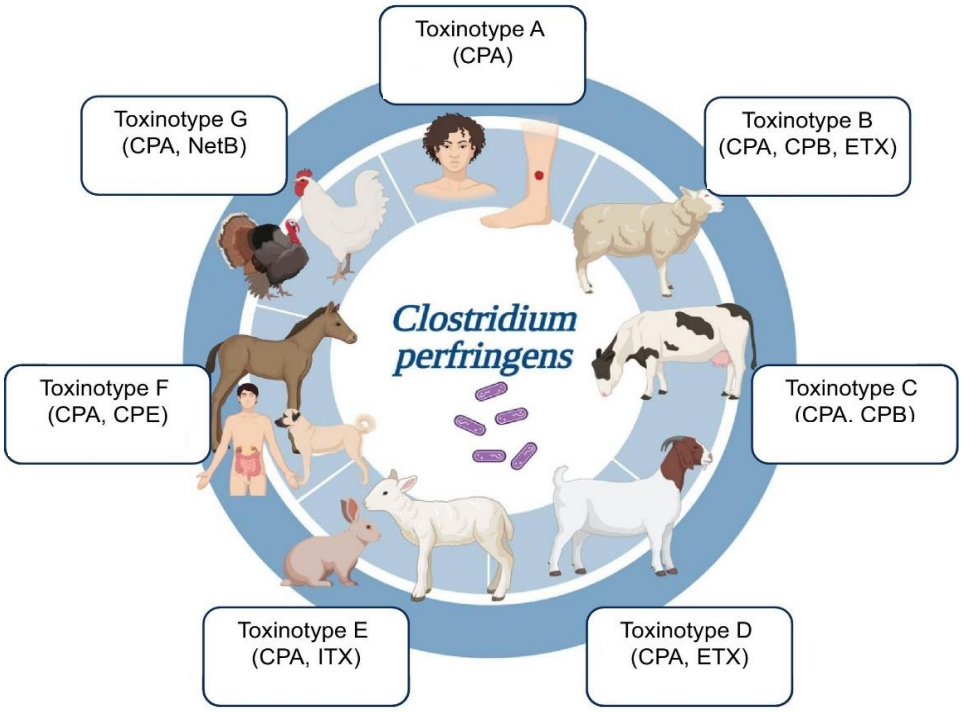


Figure 33. Host-Specific Distribution of *Clostridium perfringens* Toxinotypes **(Talukdar et al., 2025)**

Table 10. *Clostridium perfringens* Toxinotypes associated animal Diseases **(Mainil, 2006; Lebrun et al., 2010).**

Toxinotype	Hosts	Disease
A	Dogs and horses Lambs Neonates and young calves	Hemorrhagic gastroenteritis Yellow lamb disease Enteritis and enterotoxaemia
B	Lambs under week old Foals Neonates calves	Lamb dysentery Enterotoxemia, not common Enterotoxaemia with necrotic/haemorrhagic enteritis, sudden death
C	Lambs, foals and calves Piglets 1-3 days old Broiler chicken 2-12 weeks old Adult sheep and goats	Hemorrhagic enterotoxemia (clostridial enteritis) Necrotic enteritis (Types A and C) Struck
D	Sheep all ages except neonates. Young calves	Pulpy kidney disease Enteritis, with or without necrotic/haemorrhagic enteritis, sudden death
E	lambs Young calves	Enterotoxaemia Haemorrhagic enteritis and sudden death
G	Poultry	Necrotic enteritis

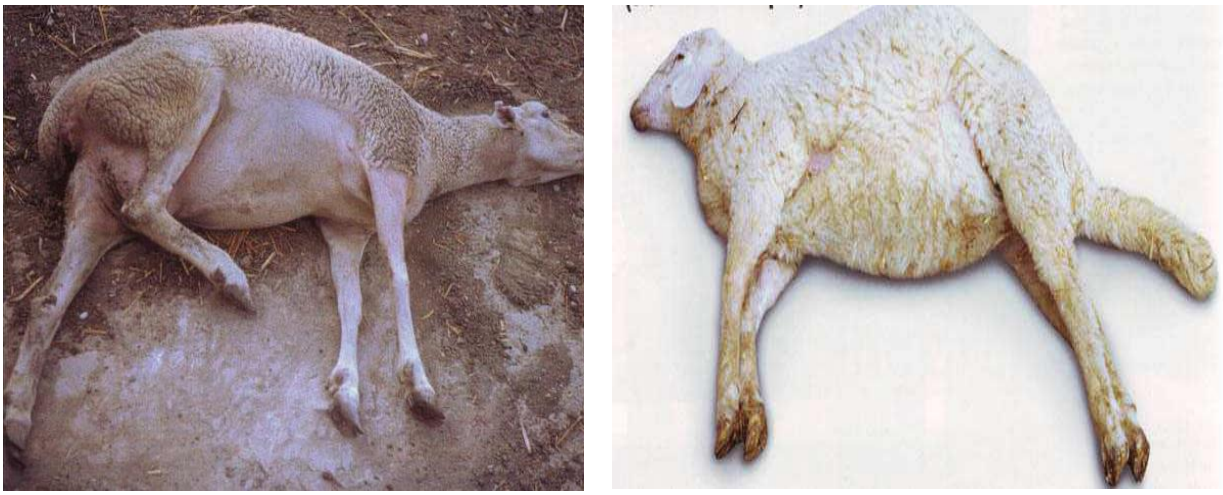


Figure 34. Acute and peracute enterotoxaemia: **On the left**, ewes showing nervous signs and an inability to stand up. **On the right**, a dead ewe lamb with tympany **(CEVA, 2009)**

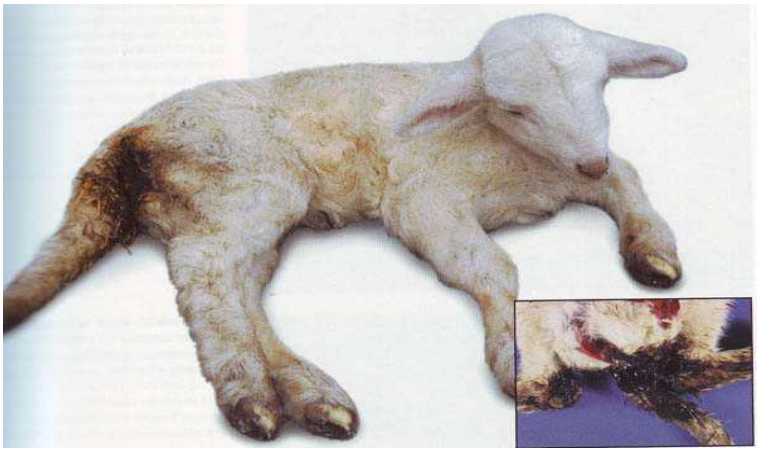


Figure 35. Haemorrhagic enterotoxaemia - Prostrate 3-day-old lamb with haemorrhagic diarrhoea with perianal area soiled with excrement **(CEVA, 2009)**

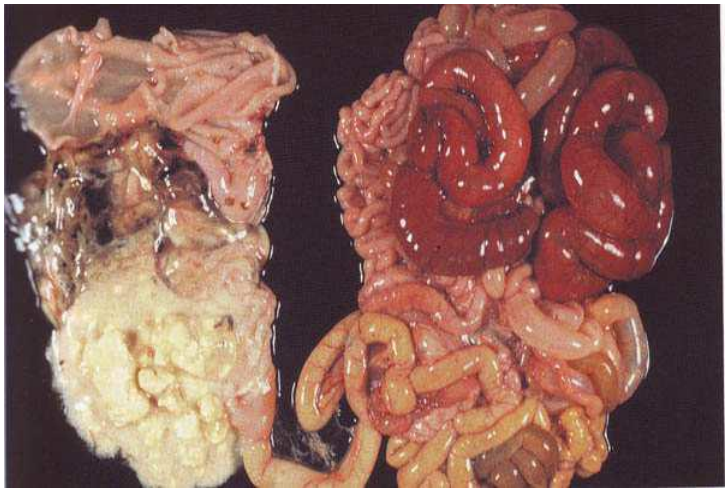


Figure 36. Haemorrhagic enterotoxaemia - Digestive system with distended intestinal tract and haemorrhagic enteritis located mainly in the jejunum. The open abomasum contains milk and remnants of blood **(CEVA, 2009)**.

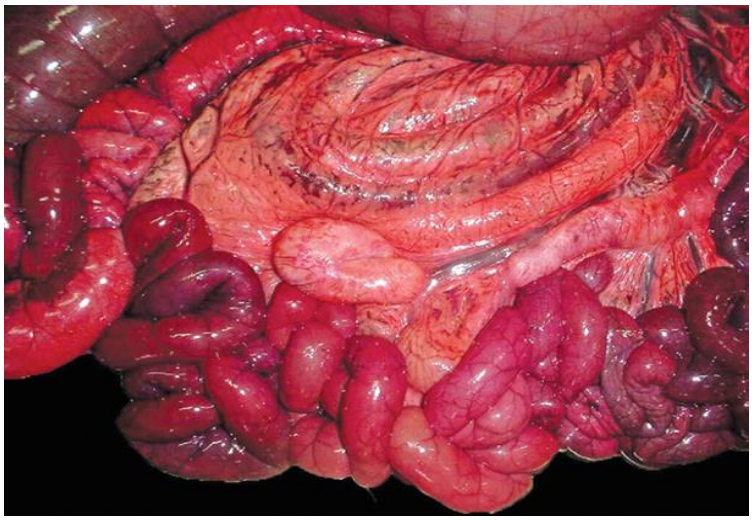


Figure 37. Diffuse hemorrhagic enteritis in a calf with *Clostridium perfringens* type B enterotoxemia **(Uzal et al., 2016)**

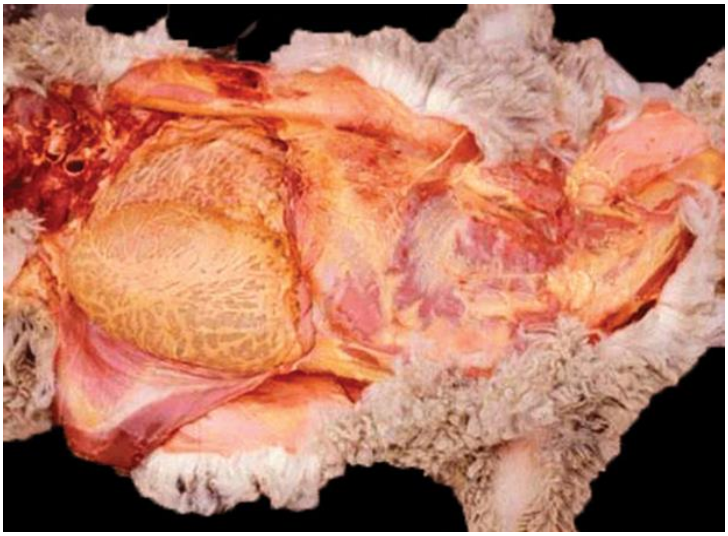


Figure 38. Sheep with yellow lamb disease showing severe, diffuse icterus **(Uzal et al., 2016)**

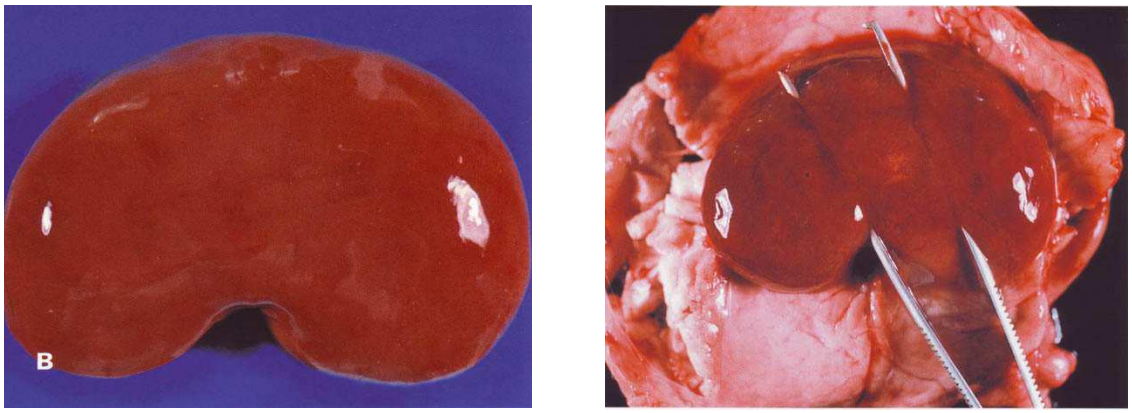


Figure 39. Pulpy kidney. This early autolysis is common in enterotoxemia caused by *Clostridium perfringens* type D enterotoxemia **(CEVA, 2009).**

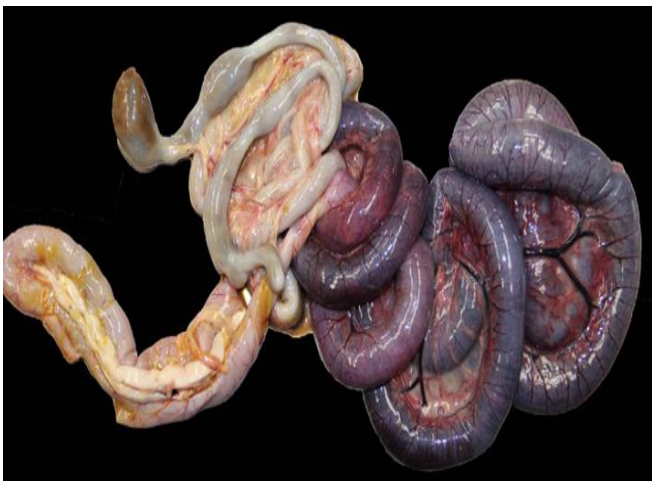


Figure 40. Serosal view of the intestinal tract of a chicken with acute necrotic enteritis, which demonstrates the predilection of the disease to develop in the small intestine, particularly the jejunum **(Uzal et al., 2016).**

II. 2.2.1.5. Laboratory diagnosis

1. Specimens

Specimens should be taken from recently dead animals because enteric anaerobic bacteria are rapid postmortem invaders. Ileal content or fecal sample (20–30 g) should be directly submitted to the laboratory for investigation. If there is a delay in transport and/or in laboratory investigation, freezing of samples at -70°C may be an alternative **(Mainil, 2006**).

2. Direct microscopy

Gram-stained smears of fecal sample or the small intestinal mucosa from recently dead animal reveals the presence of large numbers of fat gram positive rods **(Fig.40):**

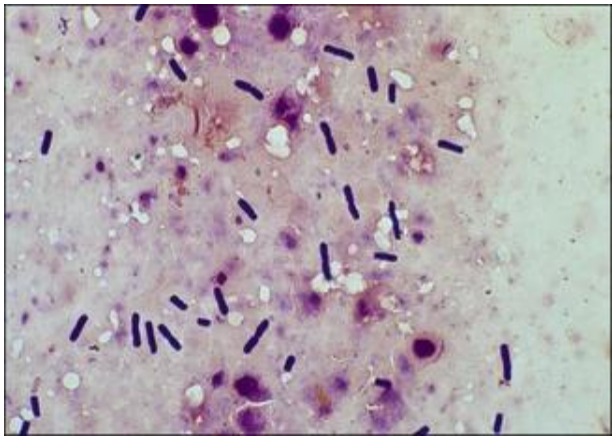


Figure 41. large Gram-positive rods *of C. perfringens* in a mucosal scraping from the small intestine of a lamb that had recently died from pulpy kidney disease (Gram stain, ×1000); **Quinn et al. (1994).**

3. Isolation

Clostridium perfringens is relatively aero-tolerant. However, it should be grown under strict anaerobic conditions in the presence of 2 to 10% CO_2 to enhance its growth. Liquid or semi solid media with a low redox potential such as cooked meat broth or thioglycollate medium can be used for selective enrichment. Immediately before inoculation, these media should be boiled to expel absorbed oxygen and rapidly cooled to 37°C **(Quinn et al., 1994**).

Isolation of *C. perfringens* can be performed then by adding the ileal content or fecal sample to pre-reduced cooked meat medium or thioglycolate medium and incubating under anerobic conditions for 24 h at 37 °C using anaerobic jar and gas packs. Then a loop of the enrichment broth is streaked on *C. perfringens* selective agar tryptose-sulfite-cycloserine (TSC) or tryptose-sulfite-neomycin (TSN) or CHROMagar™ *C. perfringens* and incubated at 37 °C for 48 hours under anaerobic conditions.

4. Identification

4.1. Colonial morphology

Typical black colonies are produced by *C. perfringens* on TSC or TSN agar due to the reduction of sulfites **(Fig. 42).** An intense orange colonies of *C. perfringens* are observed on on CHROMagar™ *C. perfringens* **(Fig. 42)**

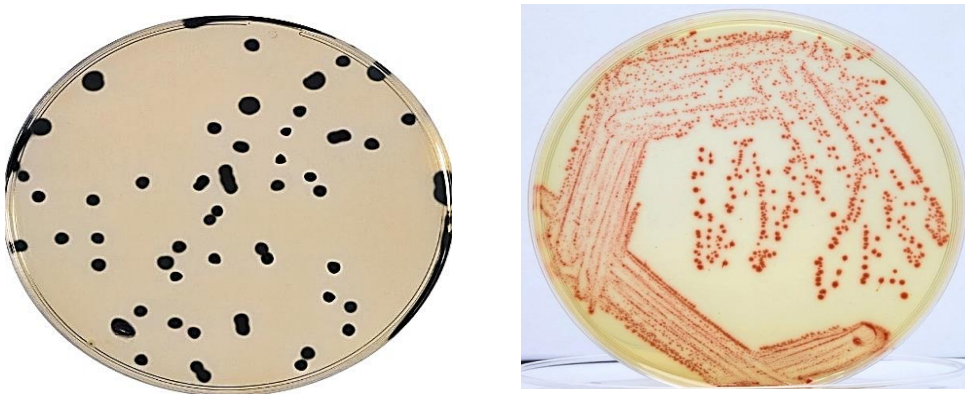


Figure 42. Colonies of *Clostridium perfringens:* on the left, black colonies on TSC agar, on the right, orange colonies on CHROMagar™ (**Mamsin et al., 2023)**.

4.2. Morphology

Short, fat , Gram positive rods that not commonly produce spores **(Fig.41).** The spores, if present, are oval, subterminal and buldge the mother cell. Chains of cells can occur **(Fig.43).**

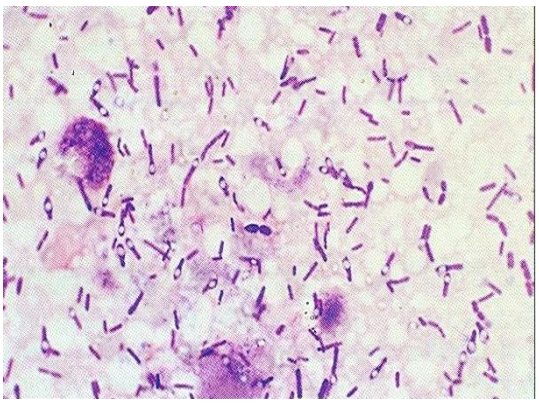


Figure 43. Spores of *C.perfringens* subterminal, oval and buldge the mother cell(Gram stain, ×1000); **Quinn et al. (1994).**

4.3.Biochemical reactions

- **Hydrolysis of casein:**

Clostridium perfringens inoculated into litmus milk medium produces the classical 'stormy-clot' or 'stormy-fermentation' reaction **(Fig.44).** The lactose in the medium is fermented by *C. perfringens* producing acid which coagulates the casein and induces a colour change from blue to pink (litmus pH indicator). The acid clot is then broken up by gas formation.

Figure 44. The 'stormy clot' reaction of three isolates of *C. perfringens* in litmus milk medium. The tube on the left is uninoculated **(Quinn et al.,1994).**

- **Nagler réaction of *C. perfringens* or lecithinase test**

Type A antitoxin (alpha toxin) is spread over half an egg yolk agar plate and allowed to dry. The suspect *C. perfringens* is streaked across both sides of the plate (all toxinotypes produce the alpha toxin, that is a lecithinase). On the half of the plate without the antitoxin, the lecithin in the medium is attacked causing opalescence around the streak. The lecithinase reaction is neutralized on the half of the plate with the antitoxin but the growth of *C .perfringens* is not affected **(Fig. 45).**

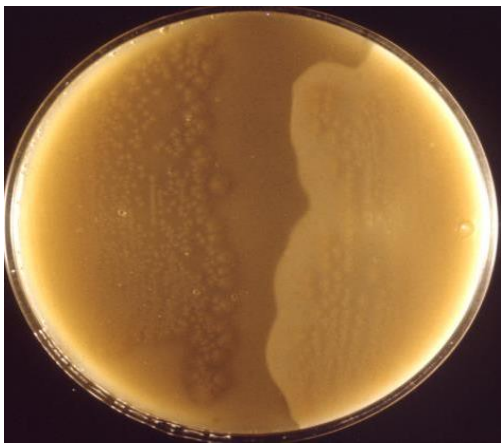


Figure 45. Nagler reaction: the toxin is a lecithinase that attaks the lecithin in the egg yolk agar (right). This reaction is neutralized on the left by specific antitoxin **(Quinn et al., 1994)**.

II. 2.2.1.6. Antibiotic susceptibility testing

Several antibiotics can be used to treat *Clostridium perfringens* infections in animals, including ampicillin, tylosin, metronidazole and erythromycin. However, antibiotic resistance is a growing concern, with some strains showing resistance to tetracycline, clindamycin, and other classes of antibiotics **(Beres et al., 2023)**.

GRAM POSITIF NON-SPORE FORMING RODS

III. 1. *Listeria* species

III.1.1. General characteristics

Listeria species are medium-sized, gram-positive, non-spore-forming rods, approximately 0.4 to 0.5µm in diameter and 0.5 to 2µm in length. They are facultatively aerobic, catalase positive, oxidase negative, hydrolyse esculin, tolerate 10% sodium chloride and are motile by a few peritrichous flagella **(Quinn et al., 1994).**

III.1.2. Classification

The genus Listeria is divided into seven species: *L. murrayi, L. grayi, L. innocua, L. seeligeri* , *L. welshimeri, L. ivanovii* and *L. monocytogenes* . The two last ones are both pathogenic for animals. However, *L. monocytogenes* is by far the most significant pathogen causing septicaemia, abortion, mastitis, and central nervous system infection in various animals, mainly ruminants. *L. monocytogenes* also causes disease in man and is an important public health concern (**Quinn et al., 1994**; **Gyles et al., 2004).**

III.1.3. Natural Habitat

Listeria species are widespread in the environment and can be isolated from soil and decaying vegetation, where they survive and grow as saprophytes. They can also be isolated from sewage, water, silage, poultry, various meats, slaughterhouse waste, raw milk and cheese.

Listeria species can grow in a temperature range of 3-45°C and in a pH range of 5.6-9.6. Silage is often implicated in outbreaks of listeriosis in cattle and sheep, as the bacteria can grow in silage with a pH above 5.5. Human foods commonly associated with human listeriosis include coleslaw, soft cheeses, deli meats, milk, hot dogs, seafood and undercooked poultry **(Quinn et al., 1994).**

L. monocytogenes has been recovered from many species of mammals, fish, birds, crustaceans and insects. Asymptomatic fecal carriers of *L. monocytogenes* occur in humans and animals. It is likely that exposure of animals to *L. monocytogenes* is unavoidable because of the many sources of the bacterium and the organism's resilience and persistence in the environment.

III.1.4. Virulence factors

Listeria monocytogenes is a facultative intracellular pathogen. Factors contributing to the virulence of this bacterium can be divided into key events: attachment-invasion, intracytoplasmic growth and cell-to-cell propulsion **(Gyles et al., 2004).**

L. monocytogenes infections are initiated by adherence or attachment to animal tissue cells. This is followed by invasion and internalisation via the internalin A proteins.

Both phagocytes and tissue cells can be infected. The bacteria escape from the phagosome via its haemolysin (listeriolysin O, LLO) and the phosphatidylinositol-dependent phospholipase C **(Glomski et al., 2002).**

Once free in the cytoplasm, intracytoplasmic replication occurs, leading to invasion of neighbouring cells. The method of cell-to-cell spread of *L. monocytogenes* is unique to this micro-organism: deposition of host cell actin filaments at the end of bacterial cells by the protein ActA promotes propulsion and invagination into neighbouring cells. The invagination process results in a double membrane around the bacterium. After invasion of new cells, the process of phagosome escape begins again with lysis of the double membrane mediated by LLO and phosphatidylinositol-dependent phospholipase C.

Repetition of the entire process leads to centripetal distribution of the microorganism **(Fig. 46):**

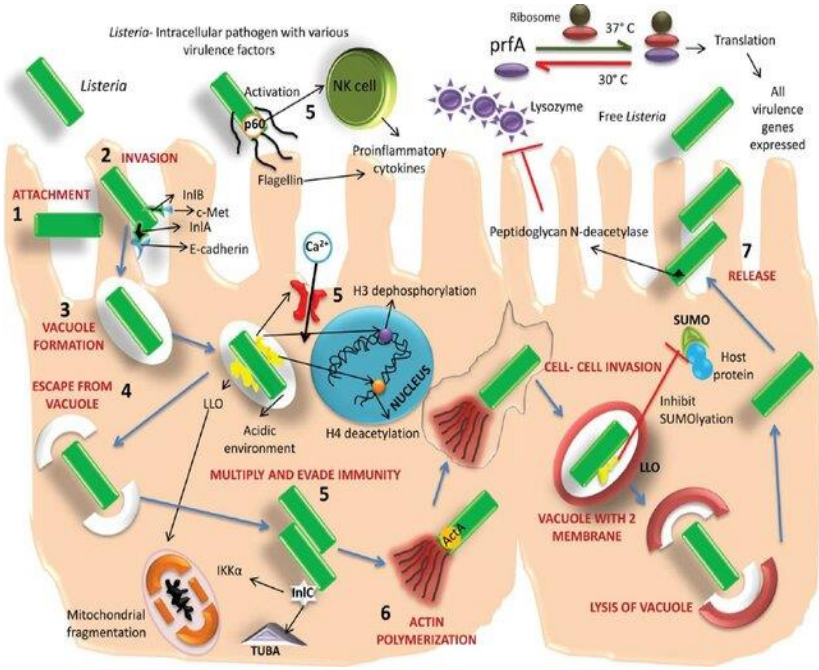


Figure 46. Virulence factors and pathogenesis of *Listeria* **(Dhama et al., 2015)**

(1) Attachment of Listeria to their receptor **(2)** Invasion **(3)** Phagocytic vacuole carries the organism inside **(4)** Listeria escapes the vacuole by several mechanisms including LLO **(5)** Listeria evades host immunity by various mechanisms including LLO, Flagellin, and InlC **(6)** Formation of actin tail propels the organism from one cell to another **(7)** Finally, release of the organism**.**

The main virulence factors of *Listeria monocytogenes* of this bacterium are summarized in Table 11:

Table 11. Main virulence factors of *Listeria monocytogenes***(Gyles et al., 2004)**

Virulence factor	Functions
Internalin A***:*** surface protein **(inlA)**	Binds to E-cadherin on the basolateral surface of intestinal epithelial cells, a process involved in the invasion of intestinal epithelial cells
Internalin B: surface protein **(inlB)**	Cell invasion
Internalin C: surface protein **(inlc)**	Cell invasion
Internalin related protein **(irpA)**	Unknown role during disseminated infection
Listeriolysin O **(LLO):** thiol-activated haemolysin	Lysis of phagosome membrane and escape into the cytoplasm
Phospholipase C **(PC-PLC)**	Mediates bacterial cell-to-cell spreadLysis of phagosome membrane and escape into the cytoplasm
Phosphatidylinositol-specific phospholipase C **(PIPL-C):** lecithinase	Mediates bacterial cell-to-cell spread, lysis of phagosome membrane and escape into the cytoplasm
Actin-polymerizing protein ActA**:** surface protein **(actA)**	Directs the deposition of host-dependent actin filaments on the end of *Listeria* cells for propulsion into neighbouring cells
Positive regulatory factor A **(prfA)**	A temperature-, pH- and nutrient-regulated factor for *Listeria* virulence determinants
Invasion-associated protein **(iap*):*** major extracellular protein (p60)	Invasion
Metalloprotease **(mpl*)***	Involved in the proteolytic activation of phospholipase C (PC-PLC)
Flagellin **(fla)**	Flagellar protein with murein-degrading activity
DegU regulator **(DegU)**	Pleiotropic regulator involved in expression of both motility at low temperature and in vivo virulence in mice

III.1.5. Pathogenesis

In most animals, *L.monocytogenes* enters into the body by penetrating the epithelial barrier in the intestine **(Fig. 47).** Subsequently, it multiplies in hepatic and splenic macrophages aided by a haemolysin, listerolysin O which disrupts lysosomal membranes allowing the organism to grow in the cytoplasm **(Quinn et al., 1994; Brugère-Picoux, 2008)**.

Bacteraemia can either be subclinical or lead to clinical septicemia. Septicaemia, with or without meningitis, occurs more commonly in neonatal ruminants and in adult sheep,

particularly in pregnant ewes and when the quantity of ingested Listeria is high. Gastrointestinal listeriosis is characterized by a marked enteritis with diarrhea (sometimes with extensive haemorrhages) and ulceration of the abomasum and intestinal mucosae.

Another possibility is the ascending infection of the trigeminal (cranial nerve V) or other cranial nerves following lesion of the buccal mucosa (trauma, shedding of deciduous or permanent teeth, periodontitis**).**

Others routes of infection have also been described, as follows: **(i)** ascending infection in the sensory nerves of the skin following dermatitis from prolonged wetting of fleece leading to listerial myelitis; **(ii)** infection of the mammary gland appears to be haematogenous, although introduction of environmental bacteria into the udder cannot be excluded; **(iii)** airborne transmission causing keratoconjonctivitis and iritis **(Brugère-Picoux, 2008)**.

Three different forms of listeriosis have been documented in animals: septicemic, encephalitic and abortion. Listeriosis causes encephalitis, abortion, mastitis, repeat breeding and endometriosis in animals. It is primarily a disease of ruminants, especially sheep, causing encephalitis and abortion. It also causes syndromes of septicemia, gastroenteritis, mastitis and conjunctivitis in ruminants. The encephalitic form is known as 'circling disease' because the animal moves in circles in one direction **(Dhama et al., 2002)**.

Occasionally, septicemic disease occurs in horses and pigs. Outbreaks of listeriosis in birds are rare and disease is occasionally observed in young chicks. Disease is sporadic/rare in poultry, usually presenting as septicemia or localized encephalitis. In addition to causing disease in domestic animals and birds, *L. monocytogenes* also affects rodents and wildlife **(OIE, 2014).**

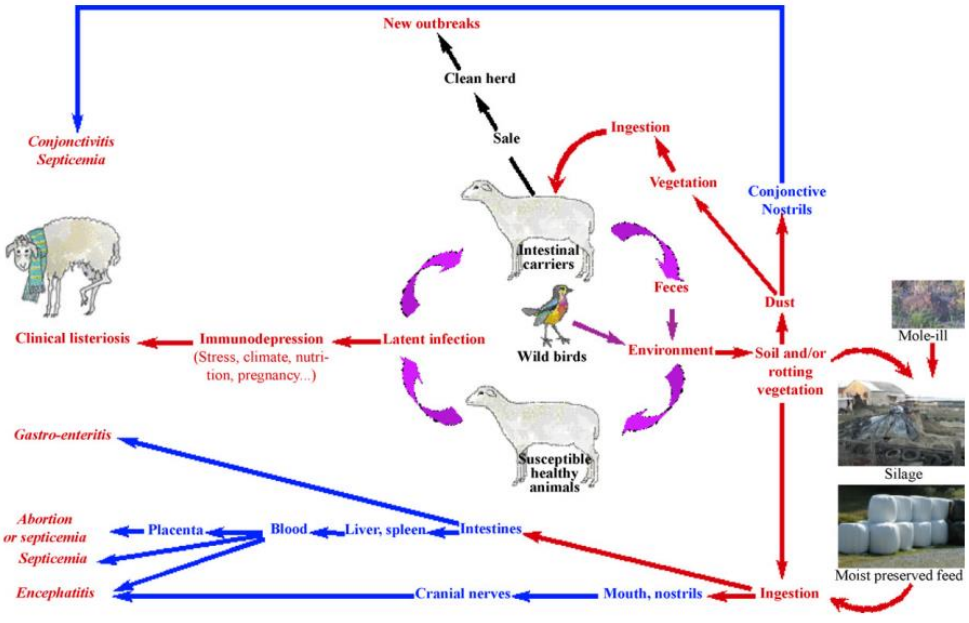


Figure 47. Etiology and pathogenesis of ovine listeriosis **(Brugère-Picoux, 2008)**.

Main hosts and disease syndromes caused by pathogenic listeria in animals are indicated in the following table:

Table12. Main hosts and disease syndromes of the pathogenic *Listeria* **(Quinn et al., 1994).**

Species	Host (s)	Disease syndromes
Listeria monocytogenes	Young animals of many species including lambs and calves. Birds can be affected	Visceral (speticemic) listeriosis: necrotic foci in liver and other abdominal organs
	Sheep, goats and cattle	Neural listeriosis (circling disease) with microabcesses in the brain - stem and pervascular cuffing.
	Sheep, goats and cattle	Abortion
	Cattle	Iritis, with or without other signs often associated with feeding big-bale silage
Listeria ivanovii	Cattle and sheep	Abortion

Figure 48. Neural form of listeriosis; **on the left**: unilateral facial paralysis in a silage-fed sheep **, on the right**: Adult sheep with right facial nerve paralysis due to listeriosis; drooped right ear, right eye showing no reaction to the flash and atony of right nostril **(Quinn et al., 1994; Brugère-Picoux, 2008)**.

Figure 49. Encephalitis due to listeriosis; **on the left**: Adult sheep marked apathy**, on the right**: Terminal stage of encephalitis in a sheep with permanent decubitus **(Brugère-Picoux, 2008)**.

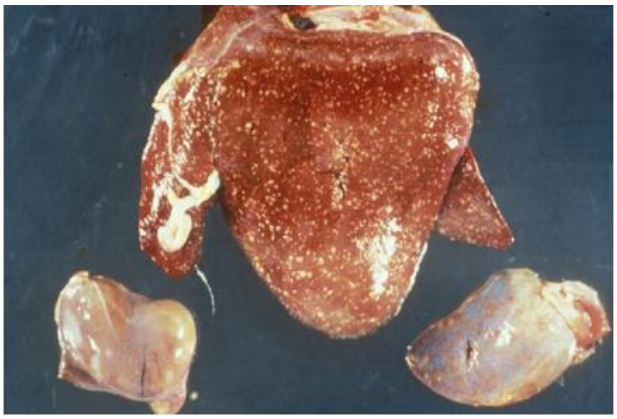


Figure 50. Post-mortem findings in a two-day-old lamb with septicaemia caused by listeriosis; presence of micro-abscesses on the heart, the liver and the kindeys **(Brugère-Picoux, 2008)**.

III.1.6. Laboratory Diagnosis

III.1.6.1. Specimens

A full range of specimens should be submitted so that an examination can be made for the other pathogens capable of causing abortion. Specimens for isolation of *Listeria* species should be cultured immediately at 35°C or stored at 4°C for up to 48 hours. To avoid overgrowth of contaminants, freezing at −20°C is recommended. Feed samples such as silage for epidemiological studies should be collected (at least 100 g) as aseptically as possible in sterile containers **(Quinn et al.,1994).**

- **Neural form:** brain stem, spinal fluid, tissue from several sites in the medulla oblongata
- **Visceral form:** material from lesions in liver, kidney or spleen.
- **Abortion**: placenta (cotyledons), amniotic fluid fœtal abomasal contents and fœtal tissues (lung, abomasum) and/or uterine discharges.

III.1.6.2. Direct microscopy

Stained smears are not as useful in cases of listeriosis as they are in some other diseases. Smears from lesions may reveal Gram-positive rods , often coccobacillary **(Fig. 51**) but isolation should always be attempted. Histopathological examination of fixed (10% formalin) brain tissue can often give a presumptive diagnosis of neural listeriosis due to the characteristic lesions (Microabvesses in the brain stem, usually unilateral **(Fig.52a)** together with perivascular cuffing **(Fig.52b)** is very characteristic of listeriosis **(Quinn et al., 1994).**

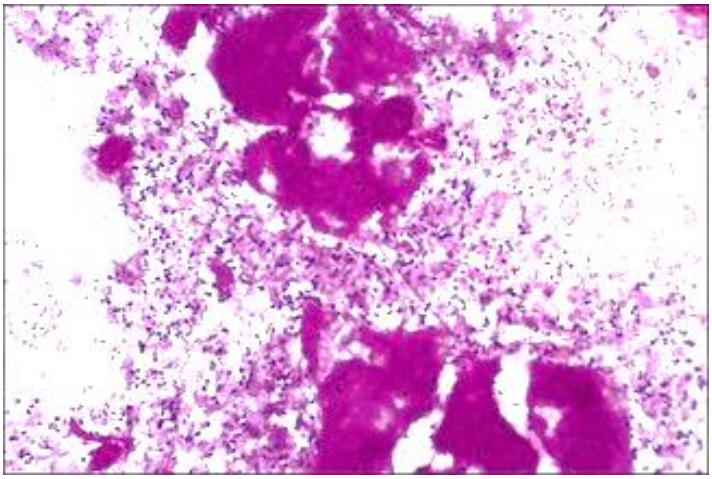


Figure 51. *Listeria monocytogenes* in a Gram-stained smear of material from a placenta **(bovine abortion), (×1000)**

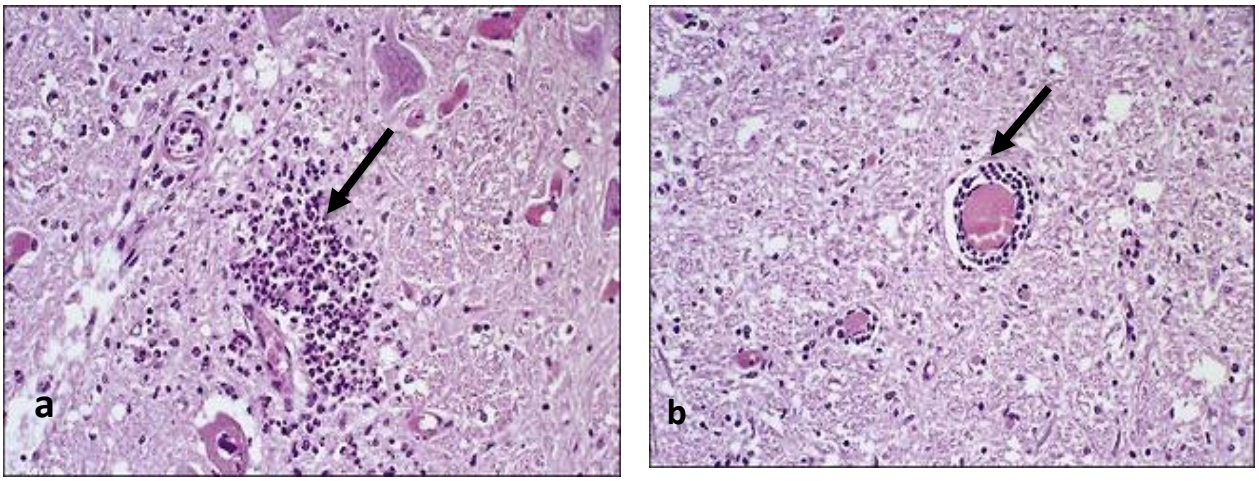


Figure 52. Characteristic microscopic lesions of neural listeriosis; **a.** Microabscess in the medulla of a sheep with listeriosis, **b.** Perivascular cuffing in an ovine medulla indicative of the neural form of listeriosis **(H&E stain,×400)**

III.1.6.3. Isolation

The routine medium for inoculation of clinical specimens is tryptic soy agar with 5% ox or sheep blood. A MacConkey agar plate may be used to detect any gram-negative pathogens or contaminants. Specimens from septicemia and abortion cases are inoculated directly onto the laboratory media and incubated aerobically at 37°C for 24 to 48 hours **(Quinn et al., 1994).**

Selective media include blood agar supplemented with antibiotics or blood agar containing 0.05% potassium tellurite, which inhibits gram-negative bacteria. A "cold enrichment" technique may be required for isolation from brain tissue in cases of neural listeriosis. This technique involves homogenizing small pieces of spinal cord and medulla and preparing a 10% suspension in a nutrient broth. The broth suspension is refrigerated at 4°C and subcultured on blood agar once a week for up to 12 weeks. This method selects for *L. monocytogenes*, one of the few pathogens that can grow at refrigerator temperature **(Quinn et al., 1994).**

The use of a selective *L. monocytogenes* enrichment method is recommended for non-sterile samples or foods to increase sensitivity and recovery of damaged Listeria cells. Selective enrichment methods involve culturing samples in a selective enrichment broth containing inhibitors, such as acriflavine and nalidixic acid, designed to slow the growth of competing organisms **(Gasanov et al., 2005)** .

A second round of enrichment is sometimes used and is referred to as two-step enrichment. After incubation, an aliquot of the broth is plated on selective agar prior to biochemical identification of typical colonies. A number of selective enrichment broths are available such as Listeria Enrichment Broth (LEB), Listeria Half Fraser Broth for primary enrichment and Listeria base Fraser Broth for secondary enrichment. Selective and indicator media are also available such as Agar Oxford, Agar PALCAM, and Agar Modified Oxford (MOX) **(Gasanov et al., 2005).**

III.1.6.4. Identification

1. Colonial characteristics

Small transparent colonies with smooth borders appear on blood agar in 24hours, becoming greyish-white and 0.5-2 mm in diameter in 48hours. *L.ivanovii* produces a comparatively wide zone of hemolysis and is very similar in appearance to a beta-hemolytic streptococcus (**Quinn et al.,1994).** *L.monocytogenes* and the non-pathogenic *L.seeligeri* have narrow zones of beta-hemolysis, often only under the colony itself.

Figure 53. *Listeria monocytogenes* on sheep blood agar, the characteristic halo surrounding individual colonies is due to β-hemolysis

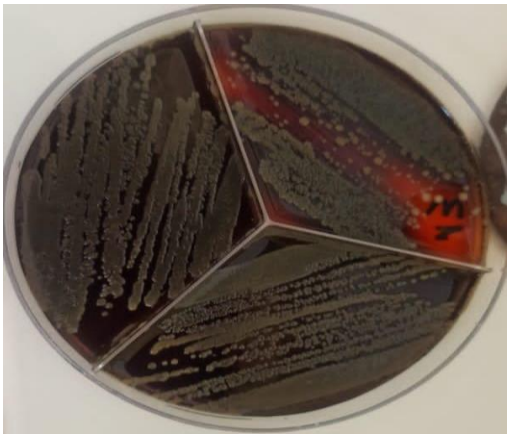


Figure 54. Black colonies of *Listeria monocytogenes* on selective agar PALCAM
Laboratory of microbiology, IVS-Tiaret

2. Microscopic appearance

Short or Gram-positive rods or coccobacilli are seen at 24hours with a tendency for cells fro; older cultures to decolorize. There are often many coccal forms in smears from young rapidly growing colonies **(Fig.55)**:

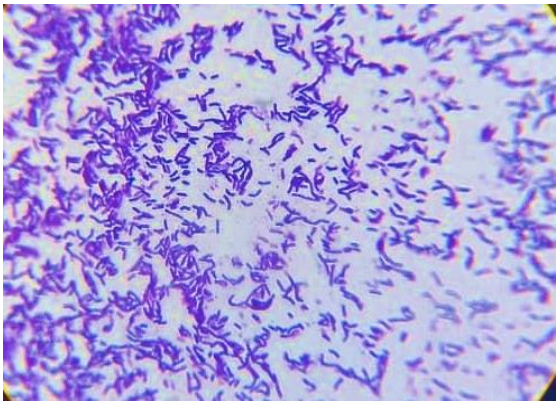


Figure 55. *L.monocytogenes* in a stained smear from a culture (Gram stain, x1000)
Laboratory of microbiology, IVS-Tiaret

3. Biochemical and other tests

- **Hydrolyse of esculin**: all Listeria species hydrolyze esculin on esculin broth, causing the medium to turn black **(Quinn et al., 1994).**
- **Motility:** *L.monocytogenes* in particular exhibits the characteristic tumbling motility when a 2 to 4 hour broth culture incubated at 25°C is examined by the hanging drop method. This motility is an end-over-end tumbling of individual cells with periods of quiescence. When grown in semi-solid motility media, the *Listeria* spp. give an unusual umbrella-shaped growth in the subsurface **(Fig.56)**

Figure 56. Umbrella motility characteristic of *L. monocytogenes* in semi-solid motility medium **(Quinn et al., 1994)**

- **CAMP Test:**

In the CAMP test, the test isolate is streaked on a sheep red blood agar plate with streaks of two other bacteria, *Staphylococcus aureus* and *Rhodococcus equi*, perpendicular to and crossing the *L. monocytogenes* streaks. After 24-48 hours of incubation at 35°C, the cross-streaks between the test organism and the *S. aureus* and *R. equi* cross-streaks intersect. As mentioned above, *L. monocytogenes* has a b-hemolysin and the zone of hemolysis around the streak is enhanced by the presence of *S. aureus* but not *R. equi*. In comparison, the hemolysin of *L. ivanovii*, the other animal pathogenic strain, is enhanced by *R. equi* but not by *S. aureus* **(Batt, 2014)**.

Figure 57. CAMP Test for Listeria showing enhancement of the staphylococcal beta-hemolysin by *L.monocytogenes* **(Hilal et al., 2017).**

- **Other tests for identification**

Commercial identification kits are a widely-used alternative to traditional biochemical testing, which is time consuming and takes a week for differentiation of species using sugar utilization tests. Test strips such as API Listeria (bio-Merieux, Marcy-Etoile, France) have been extensively validated and are now incorporated into standard methodology **(Gasanov et al., 2005).** Differentiation tests for *Listeria* species are summarized in Table13.

Table 13. Differentiation characteristics of *Listeria* species **(Batt, 2014).**

Characteristic	*L.monocytogenes*	*L. ivanovii*	*L.seeligeri*	*L.innocua*	*L.welshimeri*	*L. grayi*
β-Hemolysin	+	+	+	-	-	-
CAMP-*S. aureus*	+	-	+	-	-	-
CAMP-*R. equi*	-	+	-	-	-	-
Mannitol	-	-	-	-	-	+
Xylose	-	+	+	-	+	-
Rhamnose	+	-	+	±	±	-
Virulence	+	+	-	-	-	-

III. 2. *Corynebacterium* species and *Rhodococcus equi*

III.2.1. General characteristics

The genus *Corynebacterium* belongs to the family *Corynebacteriaceae* of the order *Actinomycetales*. Corynebacteria are gram-positive, slender, non-spore-forming bacilli. Some species may be slightly curved or have clubbed ends. Cell size can range from 0.3 to 0.8 µm in diameter and 1.5 to 8.0 µm in length. Corynebacteria are facultatively anaerobic and catalase positive. The animal pathogens are not motile. **(Betts, 2006)**

III. 2. 2. Classification

During the last 20 years, the taxonomy of these bacteria has undergone dramatic changes. Several *Corynebacterium* spp. including *Arcanobacterium pyogenes* have been placed in new genera or further differentiated into new species, and several new species have been described **(Gyles et al., 2004).** The nomenclature of *Corynebacterium* over the years is summarized in Table 14:

Table 14. Nomenclature of *Corynebacterium* species **(Quinn et al., 1994)**.

Previous name	Present name
Corynebacterium equi	*Rhodococcus equi*
Corynebacterium murium	*C. kutscheri*
Corynebacterium ovis	*C. pseudotuberculosis*
Actinomyces (Corynebacterium) pyogenes	*Trueperella (Arcanobacterium) pyogenes*
Eubacterium (Corynebacterium) suis	*Actinobaculum suis*
C. renale type I	*C. renale*
C. renale type II	*C. pilosum*
C. renale type III	*C. cystitidis*

III. 2. 3. Natural Habitat

Corynebacteria have been identified on the skin, on the mucous membranes (nasopharynx), and in the intestinal tract of both humans and animals **(Quinn et al., 1994; Gyles et al., 2004).**

- ***Corynebacterium bovis*** is considered a commensal of the bovine udder. The organism inhabits the teat canal, colonizes around the Furstenberg rosette, and is routinely isolated from clinically normal quarters, especially during lactation.
- ***Corynebacterium pseudotuberculosis*** is a commensal of the skin, mucous membranes, and gastrointestinal tract of normal sheep and a saphrophyte of sheep pen soil.
- ***Corynebacterium kutscheri*** is found in the oral and nasal cavities and in the colon and rectum of laboratory rodents, including mice, rats, hamsters, and guinea pigs.
- ***Corynebacterium renale*** is a normal inhabitant of of the lower urogenital tract.

- ***Rhodococcus equi*** is found in soil, especially in soil contaminated with horse and livestock manure. Faecal contamination increases the rate of multiplication in soil because the volatile fatty acids in faecal material promote the growth of the bacterium.

III. 2. 4. Virulence factors

Corynebacteria are pyogenic bacteria causing a variety of suppurative conditions. The pathogenic mechanism of *Corynebacteria* is closely associated with its virulence factors, including several virulence genes **(Table 15):**

Table 15. Main virulence factors of pathogenic *Corynebacterium* spp. and *Rhodococcus equi* in veterinary medicine **(Quinn et al., 1994)**

Species	Virulence factor	Mechanism of action
C. pseudotuberculosis	Phospholipase D	Increases vascular permeabiliy and facilitates dissemination, inhibition of chemotaxis, degranulation and death of neutrophils, inactivation of complement
	Cell-surface lipids	May facilitate intracellular survival
C.renale	Pili Urease Renalin (extracellular potein)	Adherence Poduction of ammonia and mucosal inflammation May facilitate lysis of host cell membrane
C.cystitidis	Pili Urease	Adherence Poduction of ammonia and mucosal inflammation
C.pilosum	Pili Urease	Adherence Poduction of ammonia and mucosal inflammation
C.ulcerans	Phospholipase D	Increases vascular permeabiliy and facilitates dissemination, inhibition of chemotaxis, degranulation and death of neutrophils, inactivation of complement
R.equi	Cell wall mycolic acid	May facilitate intracellular survival
	Capsular polysaccharides	Likely prevent phagocytosis and resistance to complement
	Exoenzymes	May promote cellular membrane destruction

III. 2. 5. Pathogenesis

The notable animal pathogens include *Corynebacterium pseudotuberculosis*, a cause of lymphadenitis and lymphangitis in small ruminants and horses, respectively, and *Corynebacterium renale*, *C. cystitidis*, and *C. pilosum*, which are opportunistic agents of urinary tract infections in cattle and occasionally other species. The primary diseases caused by the most significant pathogenic *Corynebacterium* species and *Rhodococcus equi* in veterinary medicine are outlined in Table 16.

Table 16. Major pathogenic *Corynebacterium* species and *Rhodococcus equi* animal diseases **(Quinn et al., 1994)**.

Species	Main host(s)	Disease
C. bovis	Cattle Rabbit	Clinical mastitis Abscesses
C. pseudotuberculosis	Goat, sheep, camel Horses, cattle, pigs	Caseous lymphadenitis Ulcerative lymphangitis, ventral abscessation, contagious acne (canadian horse pox)
C. kutscheri	Laboratory animals (mice, rats and guinea-pigs)	Abscesses in liver, kidneys, lungs and lymph nodes
C.renale	Cattle Pigs	Pyelonephritis and cystitis Kidneys abcessesses
C.cystitidis	Cattle	Pyelonephritis
C. pilosum	Cattle	Pyelonephritis
Rhodococcus equi	Foals (2 to 4 months old) Older foals Pigs (cattle)	Suppurative bronchopneumonia Abscesses Cervical lymphadenitis

III. 2. 5. 1. *Corynebacterium pseudotuberculosis*

Infection by *C. pseudotuberculosis* commonly occurs when the organism enters the host through superficial wounds or, less frequently, via inhalation or ingestion, where the bacteria multiply locally forming micro-abscesses, then the exotoxin phospholipase D (PLD) damages the cells membrane and therefore facilitates bacterial spread via the lymphatic system to the lymph nodes and other organs. Once within the host, the bacterium may be phagocyted by macrophages **(Barral et al., 2022).**

The PLD plays a role in disrupting the phagosome while the protein kinase G (PknG) may be responsible for the inhibition of phagosome and lysosome fusion. The copper/zinc-dependent superoxide dismutase (SodC) may inhibit the action of reactive oxygen species (ROS). The collective action of these virulence factors enables the bacteria to evade cellular clearance mechanisms, resulting in cell death following intracellular multiplication and subsequent bacterial dissemination. Other potential virulence factors include adherence pili (SpaA, SpaB, SpaC, SpaD, SpaE, and SpaF), mycolic acids (corynomycolic acids), an endo-β-N-acetylglucosaminidase (CP40), and neuraminidases (NanH), which may contribute to C. pseudotuberculosis pathogenesis **(Fig. 58).** The phagocytes disseminate the organism via the blood or lymphatic system to secondary sites. The toxicity of cell surface lipids and PLD or other factors eventually results in phagocyte lysis, with concomitant deposition of bacteria, resulting in abscessation at these secondary sites, which include the peripheral and internal lymph nodes as well as the visceral organs **(Gyles et al., 2004).**

The enhanced vascular permeability resulting from PLD's actions may also contribute to the propagation of infection, both in the immediate vicinity and via the lymphatic system. The observation that PLD can activate chemotactic factors in sheep serum to promote neutrophil migration has the potential to enhance phagocyte-mediated dissemination of the bacteria by promoting bacteria-phagocyte proximity. Furthermore, PLD facilitates the persistenceband spread of C. pseudotuberculosis within the host; a PLD knockout mutant was unable to establish a primary infection or cause chronic abscessation of the peripheral lymph nodes **(Gyles et al., 2004).**

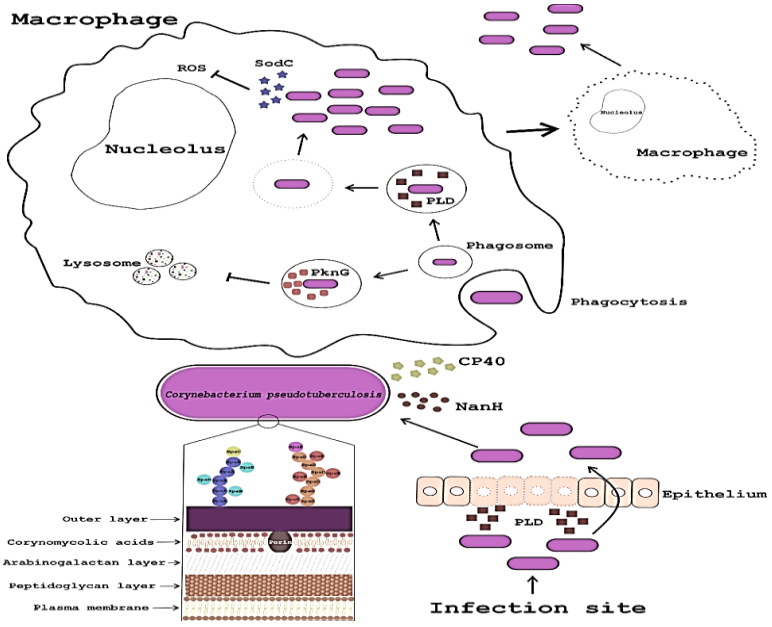


Figure 58. Scheme of virulence factors and pathogenesis of *C. pseudotuberculosis* **(Barral et al., 2022)**

GRAM POSITIF NON-SPORE FORMING RODS

Corynebacterium pseudotuberculosis is the causative agent of caseous lymphadenitis (CLA), a suppurative abscessation affecting the superficial and internal lymph nodes **(Fig. 59)** and the internal organs of small ruminants. **(Fig.60)**

Figure 59. External caseous lymphadenitis: **A.** Parotid lymph node, **B.** submandibular lesion, **C.**prescabular lymph node; **D.** prefemoral lymph node, **E.** inguinal lymph node, **F.** parotid, retropharyngeal, and sub mandibular lymph node **(Ali et al., 2016; Washburn, 2024)**

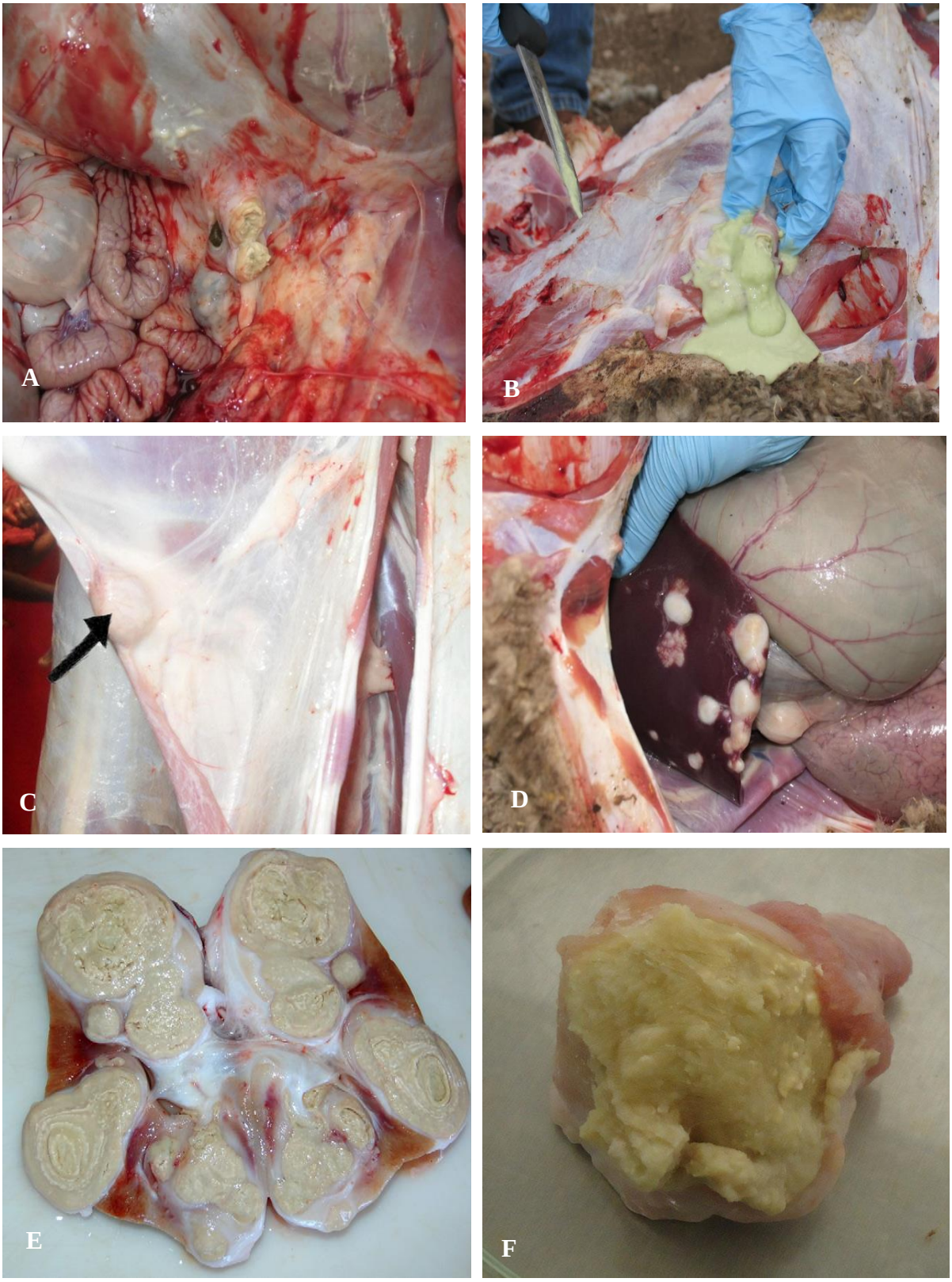


Figure 60. Internal caseous lymphadenitis: **A.** Abscesses in multiple MLN in an adult ewe, **B.** Abscess of the prefemoral lymph node of an adult goat, **C.** CLA in a prefemoral lymph node on a goat carcass, **D.** Abscesses in the liver and MLN, **E.** Visceral caseous lymphadenitis in left kidney of an adult ewe, **F.** yellow pus in an incised prefemoral lymph node affected by CLA **(Ferrer et al., 2009; Abebe & Tessema, 2015 ; Washburn, 2024)**.

Following initial entry, the organism disseminates rapidly to the local drainage lymph node. In this location, numerous microscopic pyogranulomas emerge, increasing in size and coalescing to form larger abscesses. This progression is occasionally accompanied by a subsequent dissemination of infection through the bloodstream or the lymphatic system, resulting in the manifestation of analogous lesions in disparate organs **(Baird and Fontaine**, **2007)**.

Following colonization of a lymph node by *C. pseudotuberculosis*, a brief period of generalized inflammation ensues. PLD, the soluble exotoxin produced by C. pseudotuberculosis, is the probable initiator of this lymphadenitis. Within 24 hours of subcutaneous inoculation of lambs, micro-abscesses were observed in the cortical region of the lymph node draining the site of inoculation. By day six post-inoculation, these micro-abscesses had increased in number and began to expand and coalesce, forming larger purulent foci. The initial pyogranulomas were characterized by the presence of clumps of bacteria and cellular debris, with a relatively high proportion of eosinophils, thereby imparting a slightly green hue to the purulent core **(Baird and Fontaine**, **2007)**.

Concurrently with the cellular events at the site of entry, the infiltration of neutrophils decreased, and monocytes/macrophages became the predominant cell type within the lesion. This process was accompanied by the encapsulation of the lesion, which subsequently led to a reduction in the inflammatory reaction within the node's parenchyma. The ensuing slow expansion of the lesion was often observed, contingent on the anatomical location of the node and its potential rupture, which would result in the discharge of its contents. This process of expansion and subsequent rupture is a recurring cycle that leads to the formation of a new outer capsule, a process that is often accompanied by the death of surrounding tissue (necrosis). In the early stages, the purulent contents of the abscess were soft and somewhat fluid. However, as time progressed, the pus within the lesion took on a more solid or plastic form, in which scattered clumps of bacteria were sometimes noted. The development of small nodules of mineralization within the purulent material led to a progressive paler coloration of the lesion **(Baird and Fontaine**, **2007)**.

These calcified foci were typically arranged in concentric layers, reminiscent of the cross-sectional view of an onion. **(Fig.61-63).**

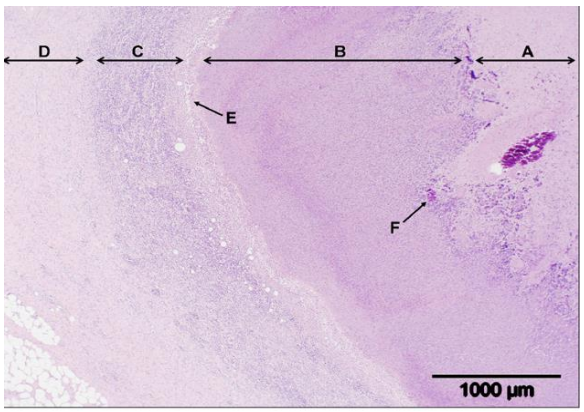


Figure 61. Transverse histological section of a prescapular lymph node from a case of ovine caseous lymphadenitis. Several distinct concentric layers are discernible within the lesion. Centrally there is liquefactive necrosis **(liquid pus; A**) which is surrounded by coagulative necrosis **(caseous pus; B),** both containing multiple foci of mineralization **(F)**, apparently in loosely arranged concentric layers. A thin layer of polymorphonuclear neutrophils surrounds the periphery of the coagulative necrosis; there is a further outer layer of coagulative necrosis containing polymorphonuclear neutrophils migrating through it at different densities, forming an apparent bi-layer **(E).** This is tightly bordered by a layer of immature fibrosis **(C)** containing mononuclear inflammatory cells. A thick layer of mature fibrosis **(D)** delineates the extent of the lesion. **(H&E**), **(Baird and Fontaine**, **2007)**.

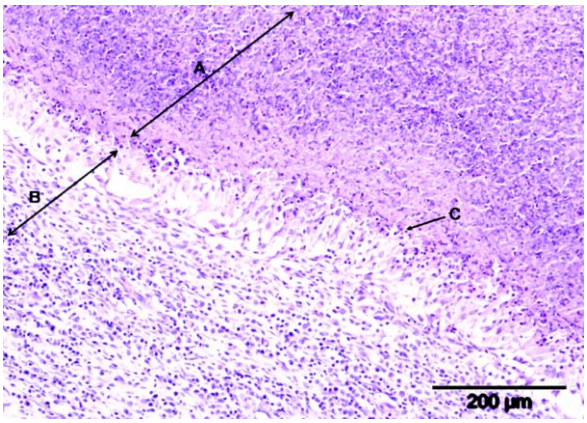


Figure 62. Higher magnification of Fig. 61, showing the border between the layers of caseous necrosis **(A)** and active immature fibrosis containing mononuclear inflammatory cells **(B).** Note the presence of polymorphonuclear neutrophils migrating across the border into the caseous necrosis **(C)** and deeper, HE **(Baird and Fontaine**, **2007)**.

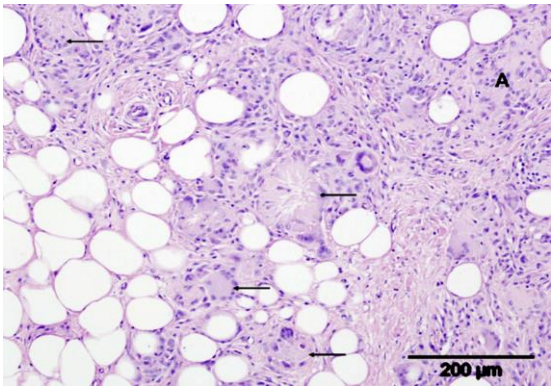


Figure 63. Transverse histological section of the interface between the outer edge of the mature fibrous capsule **(A)** and the surrounding tissue. Note the presence of many multinucleate giant cells (arrows), HE **(Baird and Fontaine**, **2007)**.

Corynebacterium pseudotuberculosis has been identified as a primary causative agent of ulcerative lymphangitis in equines **(Fig. 64a)** and chronic abscesses in the pectoral region **(Fig. 64b)**, ventral abdomen, and preputial or mammary regions

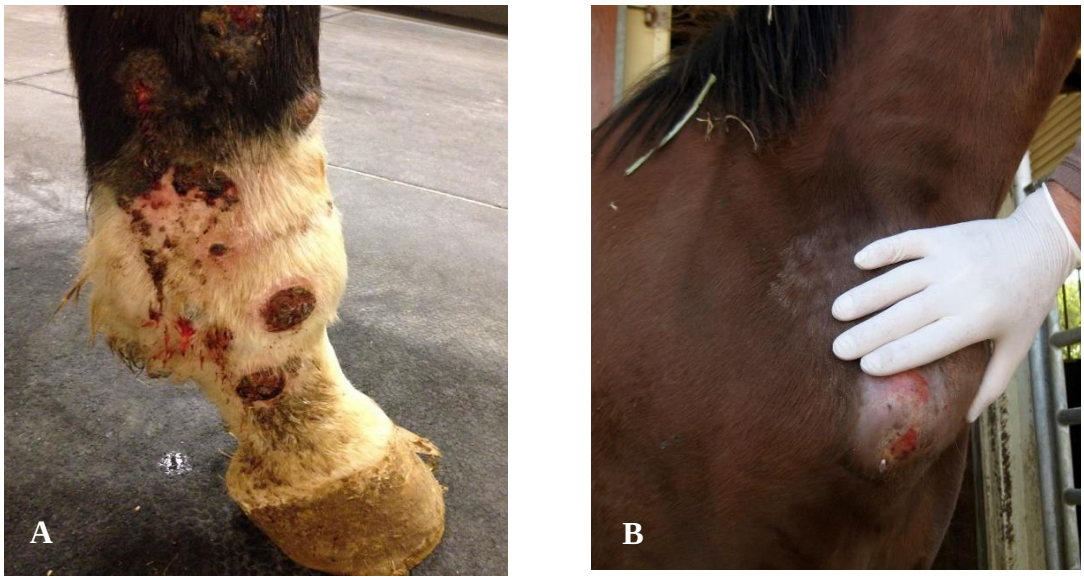


Figure 64. Equine ulcerative lymphangitis**; a.** The lesions follow a trail distally coinciding with the lymphatic chains of the distal limbs, **b.** Typical pectoral abscess due to lymphangitis (**Washburn, 2024)**

In cattle, C pseudotuberculosis most commonly manifests as cutaneous excoriated granulomas. Large ulcerative skin lesions that resemble infected granulation tissue and lymphangitis have been observed in 2%–5% of cows (Fig. 65). The location of these lesions varies, but they are often associated with skin trauma. Furthermore, abortion and mastitis may also occur(**Washburn, 2024)**

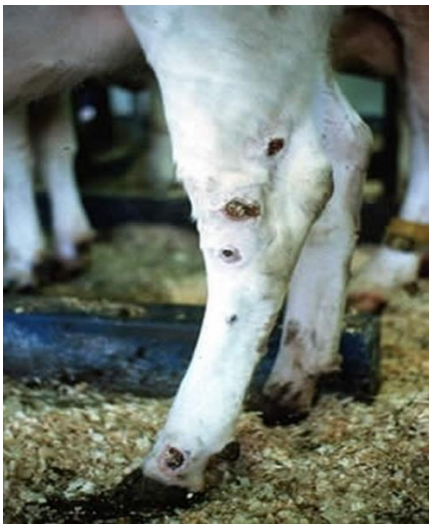


Figure 65. Bovine ulcerative lymphangitis **(Washburn, 2024)**

III. 2. 5. 2. *Corynebacterium renale* Group

Corynebacterium renale, a causative agent of bovine cystitis and pyelonephritis, was originally classified into three serologic types. Subsequent reclassification has resulted in the delineation of three distinct species: *C. renale, C. pilosum, and C. cystitidis.* These organisms can exist as normal flora in the reproductive tract of cattle; however, *C. cystitidis* is found only rarely in bulls. While all three species infect cattle, *C. renale* is by far the most prevalent and causes hemorrhagic cystitis, leading to ureteritis and pyelonephritis **(Gyles et al., 2004; Milanov et al., 2015).**

C. cystitidis is the causative agent of severe hemorrhagic cystitis, characterized by ulceration of the bladder, as well as ureteritis and pyelonephritis. In contrast, *C. pilosum* is less pathogenic, and infection results in mild, uncomplicated cystitis, although ascending pyelonephritis can occur rarely. All species from the *Corynebacterium renale* group *(C. renale, C. cystidis, and C. pilosum*) possess fimbriae that enable the attachment to the urogenital mucosa, epithelial cells of the urinary bladder, and renal pelvis. Notably, *C. renale* (the majority of isolates) is unique in its possession of an extracellular protein known as renalin. This protein has been observed to react with ceramides, which are integral components of the sphingomyelin cell wall of red blood cells in mammals. It is therefore referred to as a "renalin CAMP-like" protein because it produces synergistic hemolysis on blood agar with sphingomyelinase of *Staphylococcus aureus* (bêta-haemolysin*).* It is hypothesized that renalin plays a pivotal role in the lysis of the host cells. The enzyme urease is a significant virulence factor present in all three species from the *Corynebacterium renale* group **(Gyles et al., 2004; Milanov et al., 2015).**

Colonization is pilus-mediated, and the rapidly urease-positive nature of the organism leads to production of ammonia, with resulting mucosal inflammation **(Milanov et al., 2015).** Furthermore, it has been observed that cows with basic urine pH levels are more susceptible to developing pyelonephritis **(Fig.66).**

Figure 66. Bovine pyelonephitis associated with *Corynebacterium renale* infection (https://atlas.sund.ku.dk)

III. 2. 5. 3. *Rhodococcus equi*

The basis of the pathogenicity of *R. equi* is the ability to multiply in and eventually destroy alveolar macrophages. However, the mechanisms underlying this process are not yet fully understood. Following inhalation, *R. equi* is internalized by alveolar macrophages via the process of phagocytosis. The pathogenicity of *R. equi* is associated with its capacity to survive within macrophages, a process that involves the failure of phagosome-lysosomal fusion. This failure results in the inability of infected macrophages to perform the respiratory burst, which is essential for their function. The eventual outcome of this process is the destruction of the macrophages, due to uncontrolled intracellular replication of *R. equi* **(Gyles et al., 2004).**

Rhodococcus equi is a facultative intracellular pathogen, meaning it can survive inside macrophages to cause granulomatous inflammation. The eventual destruction of macrophages may result in the development of purulent granulomas, which can progress to caseous necrosis. In all species, the lung is the most frequently affected organ, but intestinal ulceration and lymphadenitis may follow severe intestinal infection. *R. equi* has been observed to infect wounds and disseminate from a substantial focus of infection, such as a lung, to cause abscesses throughout the body **(Prescott**, **1991).**

The susceptibility of the young foal to *R. equi* pneumonia remains largely unexplained, but must relate in part to a combination of factors, including heavy challenge by the respiratory route coinciding with declining maternally derived antibody and absence of fully competent cellular immune mechanisms. Among the potential virulence factors under consideration are capsular polysaccharide, which may impede phagocytosis of the organism, and cholesterol oxidase and phospholipase C exoenzymes ("equi factors"). Cholesterol oxidase, a prominent product of *R. equi*, is of particular interest in this regard. The collective action of these factors, when considered in unison, may confer membranolytic activity on *R. equi*. However, further elucidation is necessary to fully delineate their specific role in the context of virulence **(Prescott**, **1991).**

The synthesis of cholesterol oxidase, a pivotal enzyme in the study of rhodococci, exhibits strain-specific variations in its production. However, the correlation between this variation and virulence remains to be elucidated. The potential of mycolic acid-containing glycolipids of *R. equi* to promote granuloma formation is comparable to that of other rhodococci; however, further investigation is necessary to ascertain their role. The ability of *R. equi* to persist in and eventually destroy macrophages is the basis of its pathogenicity **(Prescott**, **1991).**

The development of clinical disease in foals is multifactorial, with microbial virulence being one contributing factor.

In addition, the antibody- and cell-mediated immunocompetency of the individual foal plays a significant role. Given its intracellular nature, *R. equi* relies on cell-mediated immunity to prevent disease. Evidence indicates that young foals exhibit a deficiency in cytotoxic T lymphocytes when compared to older foals and adult horses. It has been postulated that low concentrations of gamma interferon (IFN-γ) may serve as an additional risk factor for infection with *R. equi* **(Gyles et al., 2004).**

The primary route of pneumonic infection in foals is the inhalation of dust particles laden with virulent *R equi*. Furthermore, manure from pneumonic foals constitutes a significant source of virulent bacteria contaminating the environment. Foals with pulmonary infections have been observed to swallow sputum laden with R. equi, which has been shown to readily replicate in their intestinal tract. *R. equi* infection is characterized by a gradual progression, manifesting as acute to subacute clinical signs **(Gyles et al., 2004).**

The infection is associated with significant mortality, particularly among foals aged one to three months. Pulmonary symptoms are prevalent and consistent in foals affected with *R. equi*, and they are characterized by subacute to chronic bronchopneumonia, pyogranulomatous pneumonia **(Fig. 67),** and pulmonary abscessation **(Fig. 68).**

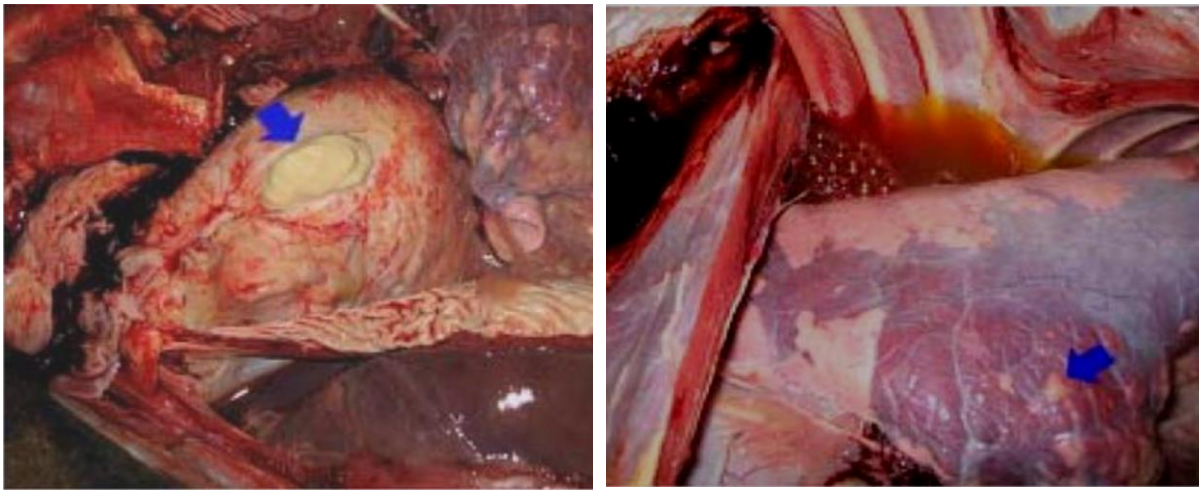


Figure 67. Lungs of the foals with extensive pyogranulomatous abscesses typical of the lesions of Rhodococcus equi pneumonia **(Kim et al., 2008).**

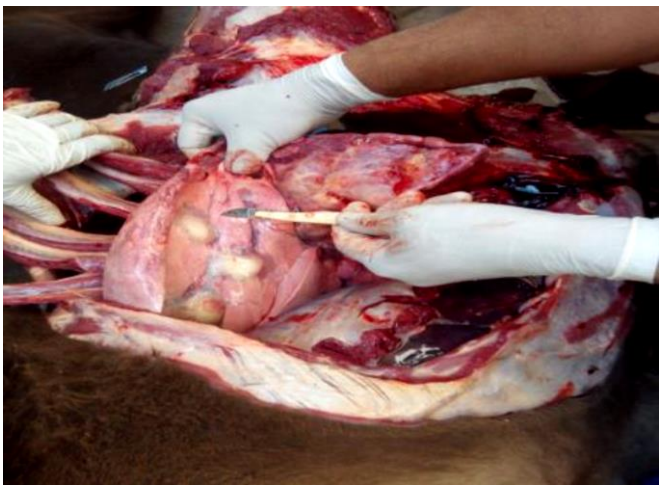


Figure 68. Multiple pulmonary abscesses characteristic of *Rhodococcus equi* (**Meena et al., 2022)**

The presence of extrapulmonary symptoms is also a possibility, with symptoms including fever, polysynovitis, uveitis, enterocolitis, abdominal abscess, and osteomyelitis **(Kumar et al., 2020).**

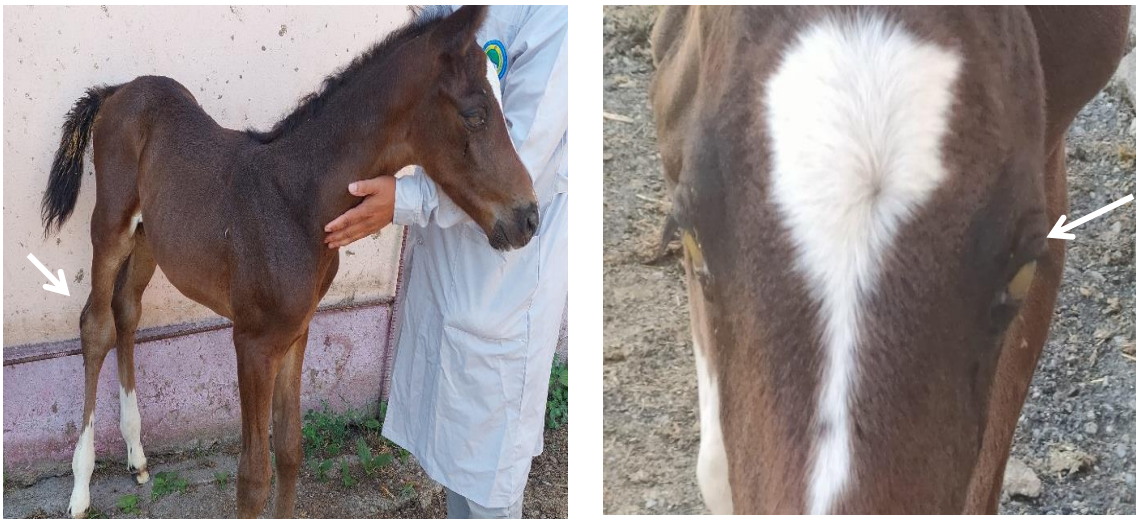


Figure 69. Foal with swelling of the joints and blurred eyes **(Ilgekbayeva et al., 2023)**

III.2.6. Laboratory Diagnosis

III.2.6.1. Specimens

Pus or exudates are collected from suppurative conditions and mid-stream urine for the attempted isolation of members of the C. renale group. A tracheal wash technique, involving the infusion of saline, can be used to recover *R. equi* from the feces of affected foals **(Quinn et al., 1994).**

III.2.6.2. Direct microscopy

The corynebacteria are Gram-positive rods that exhibit varying degrees of pleomorphism. Rhodococcus equi typically manifests as coccal forms; however, under certain conditions, such as within animal tissue, it can adopt a rod-shaped morphology. Notably, *R. equi* is MZN-positive, a characteristic indicative of its classification as a weakly acid-fast organism **(Quinn et al., 1994).**

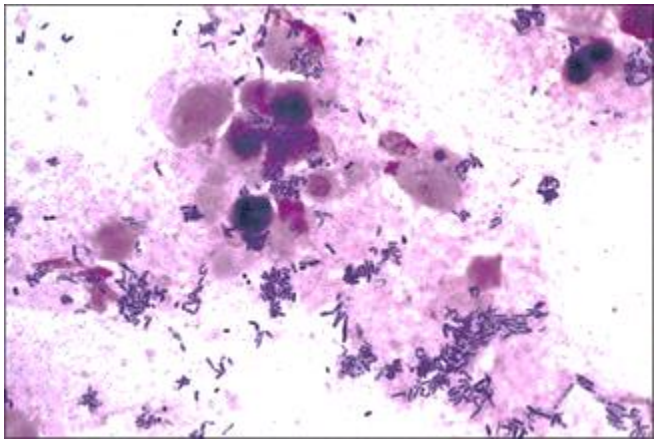


Figure 70. *Corynebacterium renale* in a Gram-stained smear of bovine urine from a case of pyelonephritis. It shows extreme pleomorphism manifesting as club-shaped rods and coccal forms (Gram stain, ×1000), **Quinn et al. (1994)**

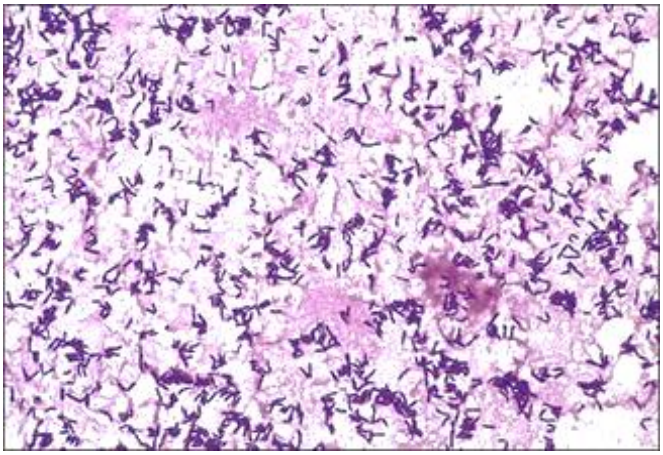


Figure 71. *Rhodococcus equi* in a smear of pus from a lung abscess in a case of suppurative bronchopneumonia in a foal. In this smear rod-shaped forms predominate (Gram stain, ×1000), **Quinn et al. (1994)**

III.2.6.3. Isolation

For routine isolation sheep or ox blood agar is used with MacConkey agar to detect any Gram-negative contaminants that may be present. The plates are incubated at 37°C for 24 to 48 hours. A selective agar medium (NANAT) is used for the isolation of *R. equi* from foal samples **(Kim et al., 2008)**.

III.2.6.4. Identification

1. Colonial morphology

- ***Corynebacterium pseudotuberculosis*** produces small, white, dry colonies. These colonies frequently exhibit a narrow zone of haemolysis surrounding them, though this is not always evident until after 48 to 72 hours of incubation. Following an extended incubation period of several days, these colonies can attain a diameter of 3 mm, exhibiting a dry, crumbly, and cream-colored appearance**.**

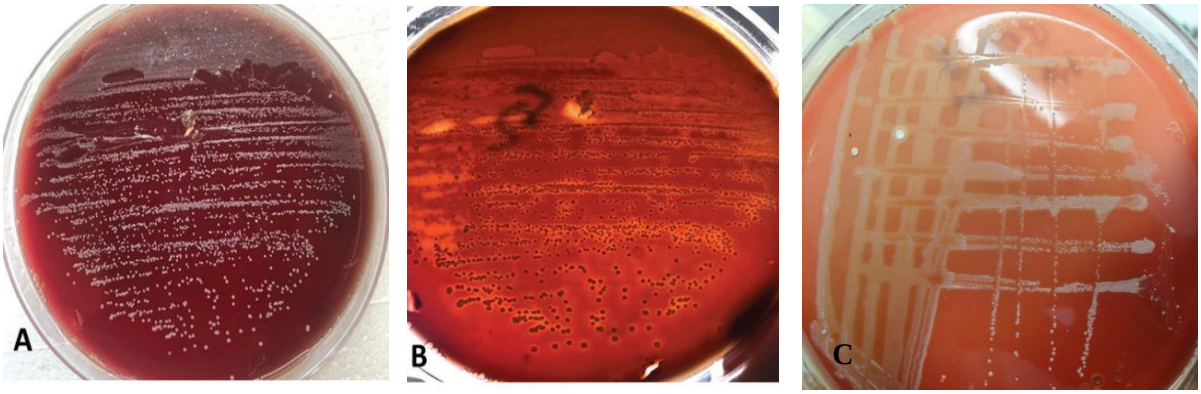


Figure 72. *Corynebacterium pseudotuberculosis* on sheep blood agar. **A.** small, white and dry non-haemolytic colonies at 24h of incubation at 37°C**, B.** Narrow and transparent zones of β-hemolysis around the colonies after 48h of incubation at 37°C**, C.** Dry, crumbly and cream colonies after 72h of incubation **(Abdulrahmanet al., 2015; Meng et al., 2023)**

- ***Corynebacterium renale*** has been shown to produce non-hemolytic small-to-medium-sized yellowish colonies following a 24-hour incubation period at 37°C on blood agar. **(Fig.73)**

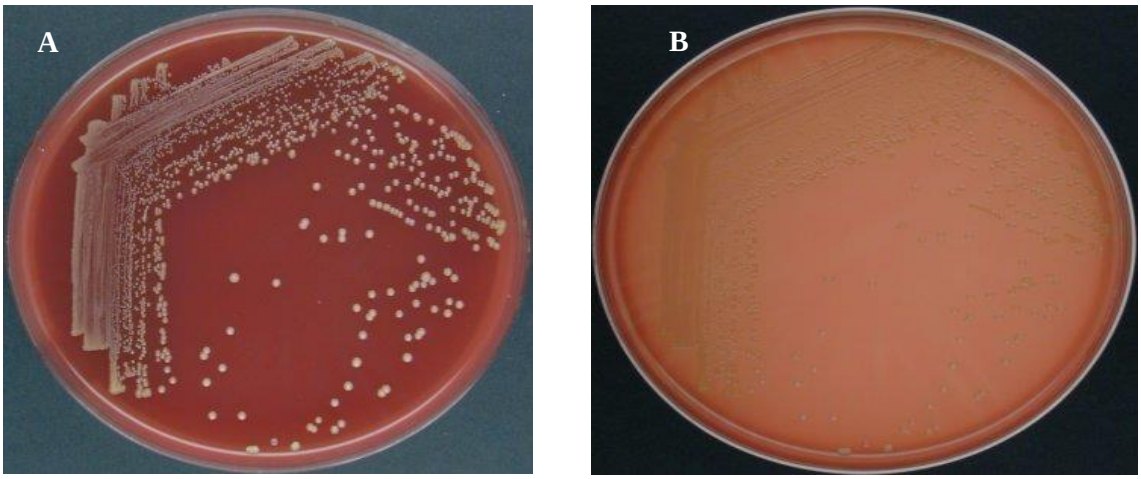


Figure 73. *Corynebacterium renale* on sheep blood agar, **A.** Small to medium sized yellowish colonies, **B.** No haemolysis on the same Blood Agar plate examined with transmitted light (https://atlas.sund.ku.dk)

- ***Rhodococcus equi*** has been observed to yield small, smooth, and shiny non-hemolytic colonies following a 24-hour incubation period at 37°C on sheep blood agar. As time progresses, these colonies undergo a transformation, becoming larger, mucoid, and salmon pink in color **(Fig.74).**

Figure 74. *Rhodococcus equi* on blood agar, **A.** Pin-point, Translucent, smooth, shiny and no hemolytic colonies after 24hr incubation, **B.** Shiny, mucoid, Salmon pink coloured colonies after 3 days of incubation at 37°C **(Alkhalifa et al., 2023)**

2. Microscopic appearance

- ***Coynebacterium pseudotuberculosis:***

Microscopic examination of Gram-stained samples revealed a large number of rod-shaped Gram-positive bacteria arranged singly and sporadically **(Fig.75)**

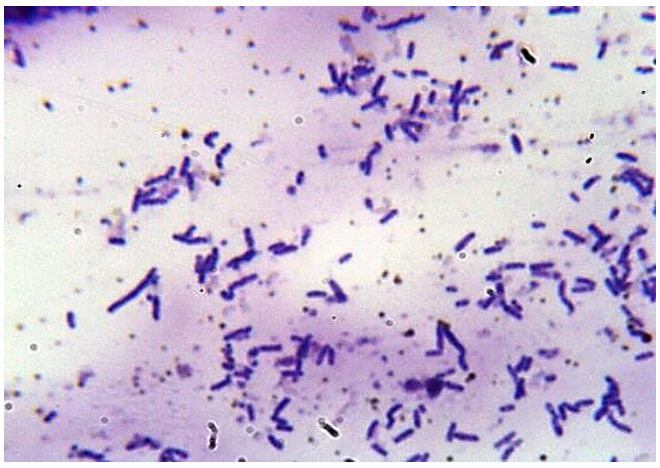


Figure 75. *Corynebacterium pseudotuberculosis* in gram-stained smear from colony showing characteristic gram-positive rods Chinese letters arrangement, (Gram stain, ×1000). **(Oreiby et al., 2013).**

- ***Coynebacterium renale:***

Gram-stained smears from colonies of C. renale typically exhibit pleomorphic gram-positive rods.

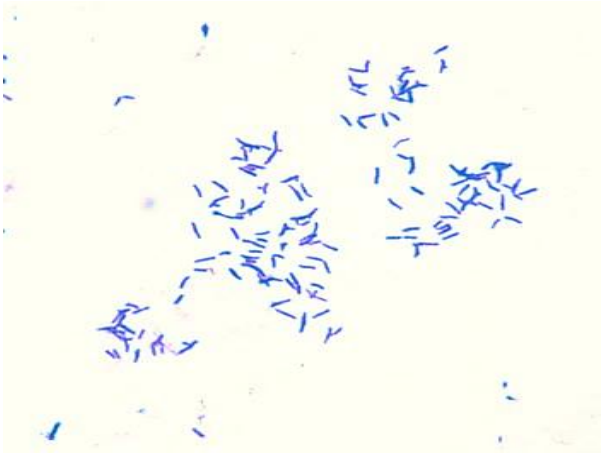


Figure 76. Gram-positive, pleomorphic rods of *C.renale* in gram stained smear of culture from bovine pyelonephritis, (Gram stain, ×1000).

- ***Rhodococcus equi:***

R. equi is a gram-positive pleomorphic coccobacillus that varies from distinctly coccoid to bacillary depending on the growth conditions **(Fig. 77).** The sporadic reports of *R. equi* being acid fast in the Ziehl-Neelsen stain appear to be contingent on the staining technique employed, the age of the cultures, and the growth medium **(Prescott, 1991)**.

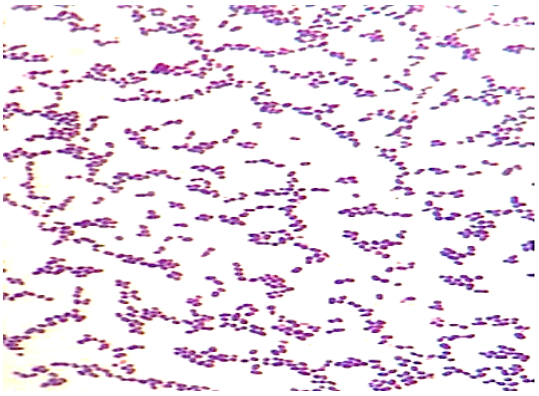


Figure 77. *Rhodococcus equi* in a Gram-stained smear from a colony with coccal forms predominating, (Gram stain, ×1000). (**Kangül et al., 2022)**

3. Biochemical tests

- **CAMP Test:** This test can be used as a quick presumptive test for *C.pseudotuberculosis*, *C.renale* and *R.equi* , interacting with the beta-hemolysin of *Staphylococcus aureus***.** The results shows the enhancement of the staphylococcal beta-hemolysin with *C.renale* and *R.equi* **(Fig.78).**

In contrast, it was reported that *C. pseudotuberculosis* exerted an inhibitory effect on the haemolysis produced by staphylococcal β-lysin, and the inhibitory agent was thought to be associated with PLD **(Baird and Fontaine, 2007).**

Figure 78. CAMP Test with *R. equi* against *S.aureus* showing shovel-shaped enhancement of the effect of staphylococcal betahemolysin that tends to extent to the opposite side of the *S.aureus* streak (**Javed et al., 2017)**.

- A synergetic hemolytic phenomenon has been seen between *Rhodococcus equi* and *Corynebacterium pseudotuberculosis* **(Fig.79).** The R. equi protein (so-called equi factor) involved in synergistic lysis was found to be a phospholipase C enzyme **(Baird and Fontaine, 2007).**

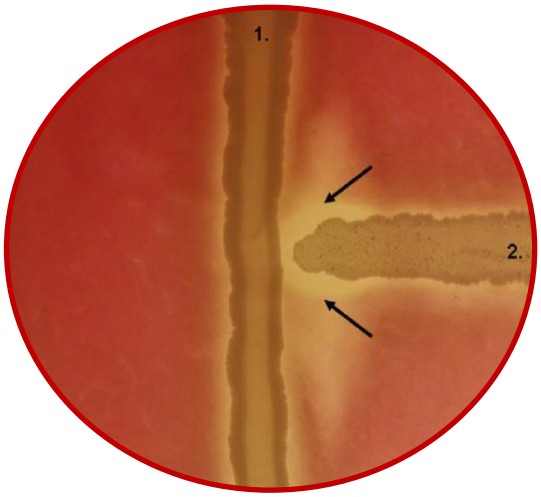


Figure 79. Synergistic lysis between *R. equi* (**1**) and C. pseudotuberculosis (**2**). The zone of enhanced lysis (**arrows**) is apparent where the soluble factors from each organism come into contact with each other **(Baird and Fontaine, 2007).**

- **Other biochemical tests**

Definitive identification of *Coynebacteria* and *R.equi* is based on differential biochemical tests (Table.17):

Table 17. Biochemical tests for differentiation of Corynebacteria and *R. equi* **(Quinn et al., 1994)**

	C.pseudotuberculosis	*Corynebacterium renale group*	*Rhodococcus equi*
β-Hemolysis	+	-	-
Aesculin hydrolysis	-	-	-
Nitrate reduction	v*	v	-
Urease	+(>18 hours)	+(<1 hour)	+(>18 hours)
Casein digestion	-	v	-
Glucose	+	+	-
Maltose	+	-	-
Sucrose	-	-	-

+: positive reaction; **-**: negative reation, **v**: variable, **v***: equine strain positive and ovine strain negative

REFERENCES

1. **Abebe, D., & Sisay Tessema, T. (2015).** Determination of Corynebacterium pseudotuberculosis prevalence and antimicrobial susceptibility pattern of isolates from lymph nodes of sheep and goats at an organic export abattoir, Modjo, Ethiopia. *Letters in Applied Microbiology*, *61*(5), 469-476.
2. **Ali, A. D., Mahmoud, A. A., Khadr, A. M., Elshemey, T. M., & Abdelrahman, A. H. (2016).** Corynebacterium Pseudotuberculosis: Disease Prevalence, Lesion Distribution, and Diagnostic Comparison Through Microbiological Culture and Molecular Diagnosis. *Alexandria Journal of Veterinary Sciences*, *51*(2).
3. **Alkhalifa, W., Ali, B. A., AlDandan, H., Aljehani, H., Alwazzeh, M., & Diab, A. (2023).** Rhodococcus equi bacteremia with necrotizing pneumonia and brain abscess in a newly diagnosed HIV patient in Saudi Arabia: A case report and review of literature. *Heliyon*, *9*(11).
4. **Baird, G. J., & Fontaine, M. C. (2007).** Corynebacterium pseudotuberculosis and its role in ovine caseous lymphadenitis. *Journal of comparative pathology*, *137*(4), 179-210.
5. **Barral, T. D., Portela, R. W., Seyffert, N., & Moore, R. J. (2022).** Corynebacterium, Arcanobacterium, and Trueperella. *Pathogenesis of Bacterial Infections in Animals*, 701-714.
6. **Betts, G. (2006)**. "Other spoilage bacteria." Food Spoilage Microorganisms, Woodhead Publishing Series in Food Science, Technology and Nutrition, 668-693
7. **Boer, M., de Voogd, M., Niemeijer, N. D., & van Hoeven, L. (2024).** Tetanus–a case report highlighting the challenges in diagnosis and treatment. *Tropical Diseases, Travel Medicine and Vaccines*, *10*(1), 10.
8. **Brugère-Picoux, J. (2008).** Ovine listeriosis. *Small Ruminant Research*, *76*(1-2), 12-20.
9. **Cai, S., Singh, B. R., & Sharma, S. (2007).** Botulism diagnostics: from clinical symptoms to in vitro assays. *Critical reviews in microbiology*, *33*(2), 109-125.
10. **CEVA Santé Animale,** Libourne, France.
11. **Dale N. Gerding, Stuart Johnson**, in Goldman's Cecil Medicine (Twenty Fourth Edition), **2012.** Clostridial infections.
12. **Dhama, K., Karthik, K., Tiwari, R., Shabbir, M. Z., Barbuddhe, S., Malik, S. V. S., & Singh, R. K. (2015).** Listeriosis in animals, its public health significance (food-borne zoonosis) and advances in diagnosis and control: a comprehensive review. *Veterinary Quarterly*, *35*(4), 211-235.

REFERENCES

13. **Deprez, P. R., Mainil, J., Duschesnes, C., Granum, P. E., & Menozzi, M. G. (2006).** Clostridia in Medical, Veterinary and Food Microbiology—Diagnosis and Typing.
14. **Dixon, T. C., Fadl, A. A., Koehler, T. M., Swanson, J. A., & Hanna, P. C. (2000).** Early Bacillus anthracis–macrophage interactions: intracellular survival and escape. *Cellular microbiology*, *2*(6), 453-463.
15. **Dlabola, J., Hashish, E., Pauly, B., Kubisiak, B., Behm, I., Heseler, R., ... & Seyboldt, C. (2015).** Clostridium botulinum type D/C intoxication in a dairy cow stock in Saxony-Anhalt (Germany)–report on an innovative diagnostic approach.
16. **Ferrer, L. M., Lacasta, D., Chacón, G., Ramos, J. J., Villa, A., Gómez, P., & Latre, M. V. (2009).** Clinical diagnosis of visceral caseous lymphadenitis in a Salz ewe. *Small Ruminant Research*, *87*(1-3), 126-127.
17. **Fraser, J., Arcus, V., Kong, P., Baker, E., & Proft, T. (2000).** Superantigens–powerful modifiers of the immune system. *Molecular medicine today*, *6*(3), 125-132.
18. **Fu, Y., Alenezi, T., & Sun, X. (2022).** Clostridium perfringens-induced necrotic diseases: an overview. *Immuno*, *2*(2), 387- 40.
19. **Gasanov, U., Hughes, D., & Hansbro, P. M. (2005).** Methods for the isolation and identification of Listeria spp. and Listeria monocytogenes: a review. *FEMS microbiology reviews*, *29*(5), 851-875.
20. **Hilal, S., Rashid, M., & Javaid, M. (2017).** Determination of prevalence and in-vitro pathogenicity of Listeria monocytogenes in sheep and goats Reared by Nomads of Jammu Region of J&K. *Journal of Animal Research*, *7*(5), 849-856.
21. **Ganesh, M., Sheikh, N. K., Shah, P., Mehetre, G., Dharne, M. S., & Nagoba, B. S. (2016).** Detection of Clostridium tetani in human clinical samples using tetX specific primers targeting the neurotoxin. *Journal of Infection and Public Health*, *9*(1), 105-109.
22. **Garcia, J. P., Anderson, M., Blanchard, P., Mete, A., & Uzal, F. A. (2013).** The pathology of enterotoxemia by Clostridium perfringens type C in calves. *Journal of Veterinary Diagnostic Investigation*, *25*(3), 438-442.
23. **Glaser, K., & Glaser, D. A. (2023).** Cosmetic Uses of Botulinum Neurotoxins for the Upper Face. *Manual of Botulinum Toxin Therapy*, 188.
24. **Goldstein, J., Morris, W. E., Loidl, C. F., Tironi-Farinatti, C., McClane, B. A., Uzal, F. A., & Fernandez Miyakawa, M. E. (2009).** Clostridium perfringens epsilon toxin increases the small intestinal permeability in mice and rats. PloS one, 4(9), e7065/

REFERENCES

25. **Gyles, C. L., Prescott, J.F., Songer, J.G., Thoen, C.O.** Pathogenesis of Bacterial Infections in Animals**,** 3rd ed. Blackwell Publishing Ltd, 2004.
26. **Hanif, H., Anjum, A., Ali, N., Jamal, A., Imran, M., Ahmad, B., & Ali, M. I. (2015).** Isolation and antibiogram of Clostridium tetani from clinically diagnosed tetanus patients. *The American Journal of Tropical Medicine and Hygiene*, *93*(4), 752.
27. **Hunter, S. E., Brown, J. E., Oyston, P. C., Sakurai, J., & Titball, R. W. (1993).** Molecular genetic analysis of beta-toxin of Clostridium perfringens reveals sequence homology with alpha-toxin, gamma-toxin, and leukocidin of Staphylococcus aureus. *Infection and immunity*, *61*(9), 3958-3965.
28. **Ilgekbayeva, G. D., Otarbaev, B. K., Kasimov, Y. I., Zanilabdin, M., Musaeva, G. K., Serikzhan, K., & Ilgekbayeva, A. K. (2023).** Biological characteristics of Rhodococcus equi isolated in the Republic of Kazakhstan. *HERALD OF SCIENCE OF S SEIFULLIN KAZAKH AGRO TECHNICAL RESEARCH UNIVERSITY: Veterinary sciences*, (2 (002)), 27-35.
29. **Javed, R., Taku, A. K., Sharma, R. K., & Badroo, G. A. (2017).** Molecular characterization of *Rhodococcus equi* isolates in equines. *Veterinary World*, *10*(1), 6.
30. **Katz, D. S. (2010).** Coagulase test protocol. *American Society for Microbiology Laboratory Protocols. Available online: https://www. asmscience. org/content/education/protocol/protocol*, *3220*.
31. **Katz, D. S. (2010).** Coagulase test protocol. *American society for microbiology*, *11*.
32. **Kim, S. J., Yook, S. Y., Hwang, J. S., You, M. J., & Jun, M. H. (2008).** Rhodococcus equi pneumonia in foals in Gyeonggi-do and characterization of the isolates from lesions and environment. *Korean Journal of Veterinary Research*, *48*(2), 139-143.
33. **Kim, H. K., Emolo, C., DeDent, A. C., Falugi, F., Missiakas, D. M., & Schneewind, O. (2012).** Protein A-specific monoclonal antibodies and prevention of Staphylococcus aureus disease in mice. *Infection and immunity*, *80*(10), 3460-3470.
34. **Kim, H. K., Emolo, C., DeDent, A. C., Falugi, F., Missiakas, D. M., & Schneewind, O. (2012).** Protein A-specific monoclonal antibodies and prevention of Staphylococcus aureus disease in mice. Infection and immunity, 80(10), 3460-3470.
35. **Kumar, S., Dedar, R. K., & Gupta, K. K. (2020).** Rhodococcus equi infection in foals–an update. *Indian J Vet Med*, *40*, 1-10.
36. **Le Maréchal, C., Woudstra, C., & Fach, P. (2016).** Botulism. *Clostridial diseases of animals*, 303-330.

REFERENCES

37. **Lebrun, M., Mainil, J. G., & Linden, A. (2010).** Cattle enterotoxaemia and Clostridium perfringens: description, diagnosis and prophylaxis. *Veterinary Record*, *167*(1), 13-22.
38. **Lee, K. W., & Lillehoj, H. S. (2021).** Role of Clostridium perfringens necrotic enteritis B-like toxin in disease pathogenesis. *Vaccines*, *10*(1), 61. https://doi.org/10.3390/vaccines10010061
39. **Le Maréchal, C., Woudstra, C., & Fach, P. (2016).** Botulism. *Clostridial diseases of animals*, 303-330.
40. **Leppla, S. H. (1991).** Purification and characterization of adenylyl cyclase from Bacillus anthracis. In *Methods in enzymology* (Vol. 195, pp. 153-168). Academic Press.
41. **Li, J., Sayeed, S., Robertson, S., Chen, J., & McClane, B. A. (2011).** Sialidases affect the host cell adherence and epsilon toxin-induced cytotoxicity of Clostridium perfringens type D strain CN3718. *PLoS pathogens*, *7*(12), e1002429.
42. **MacInnes, J. I., Van Immerseel, F., Boyce, J. D., Rycroft, A. N., & Vázquez-Boland, J. A. (2022).** *Pathogenesis of bacterial infections in animals*. J. F. Prescott (Ed.). John Wiley & Sons, Incorporated.
43. **Mainil, J. (2006).** Genus Clostridium-Clostridia in medical, veterinary and food microbiology: Diagnosis and typing.
44. **Mamsin, A. M. S., Barzani, K. K. M., & Mohammed, B. T. (2023).** Genotyping and Phylogenetic Analysis of Clostridium perfringens Isolated from Domesticated Ruminants in Duhok Governorate, Iraq. *Egyptian Journal of Veterinary Sciences*, *54*(6), 1215-1226.
45. **Maren von Köckritz-Blickwede (2009).** A Pathogen's Swiss Army Knife Posted on March 02, 2009 at 09:30 AM in Pathogens, Teachers Corner | Permalink. https://schaechter.asmblog.org/schaechter/2009/03/a-pathogens-swiss-army-knife.html
46. **Markey, B., Leonard, F., Archambault, M., Cullinane, A., & Maguire, D. (2013).** *Clinical Veterinary Microbiology E-Book: Clinical Veterinary Microbiology E-Book*. Elsevier Health Sciences.
47. **Meena, S., Bhardwaj, B & Arora, S. (2022).** A case report-isolation and identification of *Rhodococcus equi* from heart blood and tissue samples in foal. *The Pharma Innovation Journal*, SP-11(7): 2503-2505.
48. **Mehdizadeh Gohari, I., A. Navarro, M., Li, J., Shrestha, A., Uzal, F., & A. McClane, B. (2021).** Pathogenicity and virulence of Clostridium perfringens. *Virulence*, *12*(1), 723-753.

REFERENCES

49. **Meng, W., Chen, S., Huang, L., Yang, J., Zhang, W., Zhong, Z., ... & Peng, G. (2023).** Isolation, characterization, and pathogenicity assessment of Corynebacterium pseudotuberculosis biovar equi strains from alpacas (Vicugna pacos) in China. *Frontiers in Microbiology*, *14*, 1206187.
50. **Milanov, D., Velhner, M., Suvajdžić, L., & Bojkovski, J. (2015).** Corynebacterium renale cystitis in cow: Case report. *Arhiv veterinarske medicine*, *8*(1), 59-66.
51. **Mistretta, N., Brossaud, M., Telles, F., Sanchez, V., Talaga, P., & Rokbi, B. (2019).** Glycosylation of Staphylococcus aureus cell wall teichoic acid is influenced by environmental conditions. *Scientific reports*, *9*(1), 3212.
52. **Mistretta, N., Brossaud, M., Telles, F., Sanchez, V., Talaga, P., & Rokbi, B. (2019).** Glycosylation of Staphylococcus aureus cell wall teichoic acid is influenced by environmental conditions. *Scientific reports*, *9*(1), 3212.
53. **Monturiol-Gross, L., Flores-Díaz, M., Campos-Rodríguez, D., Mora, R., Rodríguez-Vega, M., Marks, D. L., & Alape-Girón, A. (2014).** Internalization of C lostridium perfringens α-toxin leads to ERK activation and is involved on its cytotoxic effect. *Cellular microbiology*, *16*(4), 535-547.
54. **Navarro, M. A., McClane, B. A., & Uzal, F. A. (2018).** Mechanisms of action and cell death associated with Clostridium perfringens toxins. *Toxins*, *10*(5), 212. doi: 10.3390/toxins10050212
55. **O'Riordan, K., & Lee, J. C. (2004).** *Staphylococcus aureus* capsular polysaccharides. Clinical microbiology reviews, 17(1), 218–234. doi:10.1128/cmr.17.1.218-234.2004
56. **Oda, M., Terao, Y., Sakurai, J., & Nagahama, M. (2015).** Membrane-binding mechanism of Clostridium perfringens alpha-toxin. *Toxins*, *7*(12), 5268-5275.
57. **Oreiby, A. F., Osman, S. A., Hegazy, Y., Ghanem, Y. A., & Al-Gaabary, M. H. (2013).** Caseous lymphadenitis in small ruminants: Descriptive, epidemiological and clinical studies. *Kafrelsheikh Veterinary Medical Journal*, *11*(1), 41-61.
58. **Pelczar Jr., M., Chan, E., & Krieg, N. (2007).** *Microbiology* (5th edition). Tata McGraw-Hill.
59. **Prescott, J. F. (1991).** Rhodococcus equi: an animal and human pathogen. *Clinical microbiology reviews*, *4*(1), 20-34.
60. **Projan, S. J. (1997).** The molecular basis of pathogenicity: Staphylococcus aureus in human diseases. *The staphylococci in human disease*, 55-81.

REFERENCES

61. **Quinn P, Carter M, Markey B, Carter G**. Clinical Veterinary Microbiology. London: Wolfe Publishing; 1994.
62. **Rawson, A. M., Dempster, A. W., Humphreys, C. M., & Minton, N. P. (2023).** Pathogenicity and virulence of Clostridium botulinum. *Virulence*, *14*(1), 2205251.
63. **Swoboda, J. G., Campbell, J., Meredith, T. C., & Walker, S. (2010).** Wall teichoic acid function, biosynthesis, and inhibition. *Chembiochem : a European journal of chemical biology*, *11*(1), 35–45. https://doi.org/10.1002/cbic.200900557
64. **Talukdar, P. K., Banawas, S., & Sarker, M. R. (2024).** Clostridium perfringens. *Trends in Microbiology*. doi: 10.1016/j.tim.2024.09.009.
65. **Tanabe, T., Sato, H., Kuramoto, M.., & Saito, H. (1993).** Purification of exfoliative toxin produced by Staphylococcus hyicus and its antigenicity. *Infection and immunity*, *61*(7), 2973-2977.
66. **Theoret, J. R., Li, J., Navarro, M. A., Garcia, J. P., Uzal, F. A., & McClane, B. A. (2018).** Native or proteolytically activated NanI sialidase enhances the binding and cytotoxic activity of Clostridium perfringens enterotoxin and beta toxin. *Infection and Immunity*, *86*(1), 10-1128.
67. **Titball, R. W., Naylor, C. E., & Basak, A. K. (1999).** The Clostridium perfringensα-toxin. *Anaerobe*, *5*(2), 51-64.
68. **Trasi Srilata, S. (2006).** Botulinum. In N. Sehgal Virendra & Srivastava Govind (Eds.), Dermatologic Surgery Made Easy (p.22). Jaypee Brothers Medical Publishers (P) Ltd. doi:10.5005/jp/books/10199_10
69. **Uzal, F. A. (2004).** Diagnosis of Clostridium perfringens intestinal infections in sheep and goats. Anaerobe, 10(2), 135-143**.**
70. **Uzal, F. A., Vidal, J. E., McClane, B. A., & Gurjar, A. A. (2010).** Clostridium perfringens toxins involved in mammalian veterinary diseases. *The open toxinology journal*, *2*, 24.
71. **Uzal, F. A. (2016).** Diseases produced by Clostridium perfringens type A in mammalian species. *Clostridial Diseases of Animals*, 107-116**.**
72. **Uzal, F. A., Songer, J. G., Prescott, J. F., & Popoff, M. R. (Eds.).** (2016). *Clostridial diseases of animals*. John Wiley & Sons.
73. **Vossen, M. G., Gattringer, K. B., Wenisch, J., Khalifeh, N., Koreny, M., Spertini, V., ... & Thalhammer, F. (2012).** The first case (s) of botulism in Vienna in 21 years: A case report. *Case Reports in Infectious Diseases*, *2012*(1), 438989.

REFERENCES

74. **Washburn, K. (2024)**. **Caseous Lymphadenitis of Sheep and Goats.** Department of Large Animal Clinical Sciences, School of Veterinary Medicine & Biomedical Sciences, Texas A&M University**.** https://www.msdvetmanual.com/circulatory system/lymphadenitis-and-lymphangitis-of-large-animals/caseous-lymphadenitis-of-sheep-and-goats.

Websites

- https://atlas.sund.ku.dk/microatlas/veterinary/bacteria/Corynebacterium_renale/
- https://veteriankey.com/the-genus-corynebacterium/
- https://veteriankey.com/listeriaspecies/#:~:text=Listeria%20species%20can%20grow%20in, with%20a%20pH%20over%205.5.
- https://www.msdvetmanual.com/circulatory-system/lymphadenitis-and-lymphangitis-of-large-animals/lymphangitis-of-horses-and-cattle